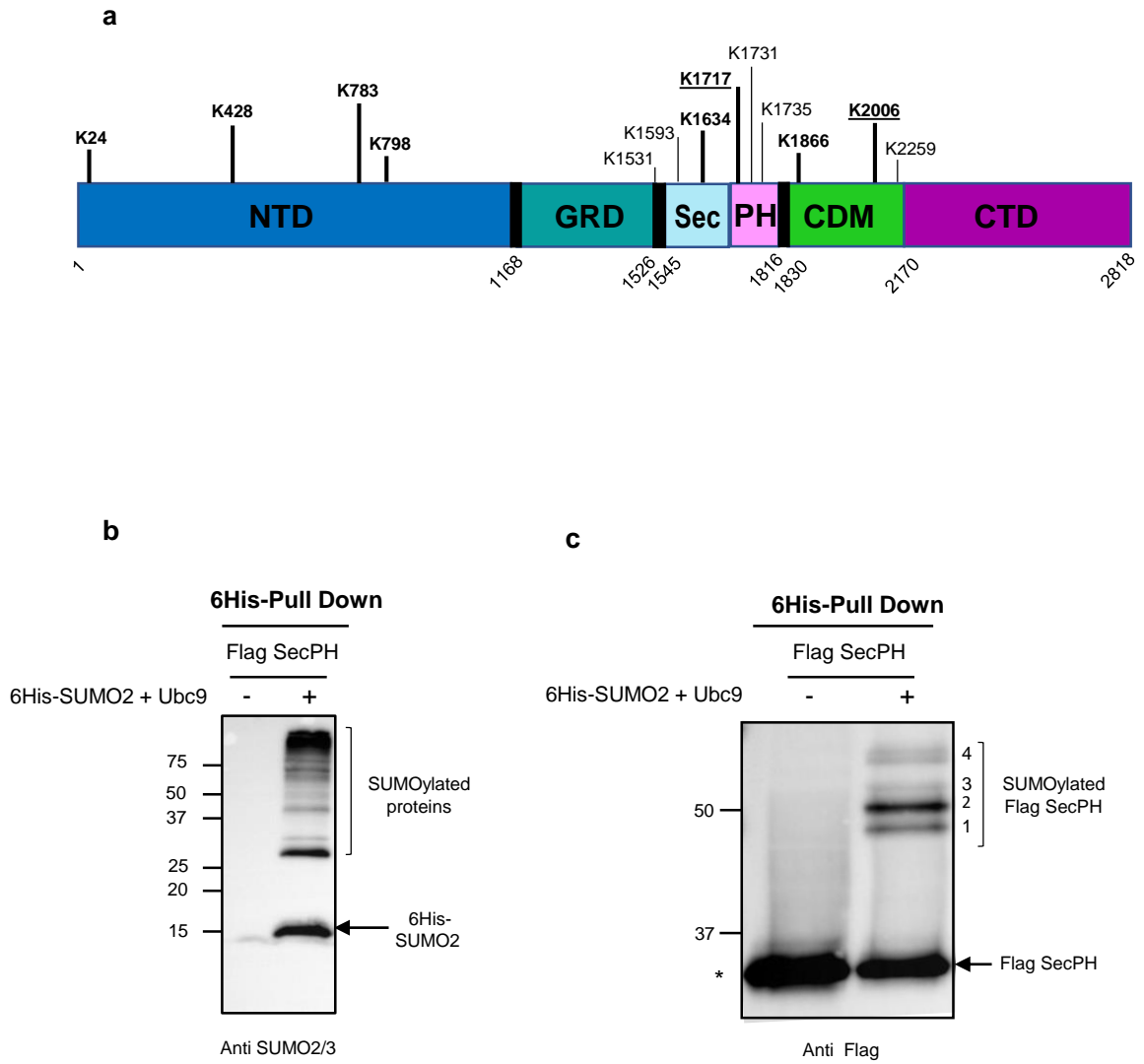


Figure 1



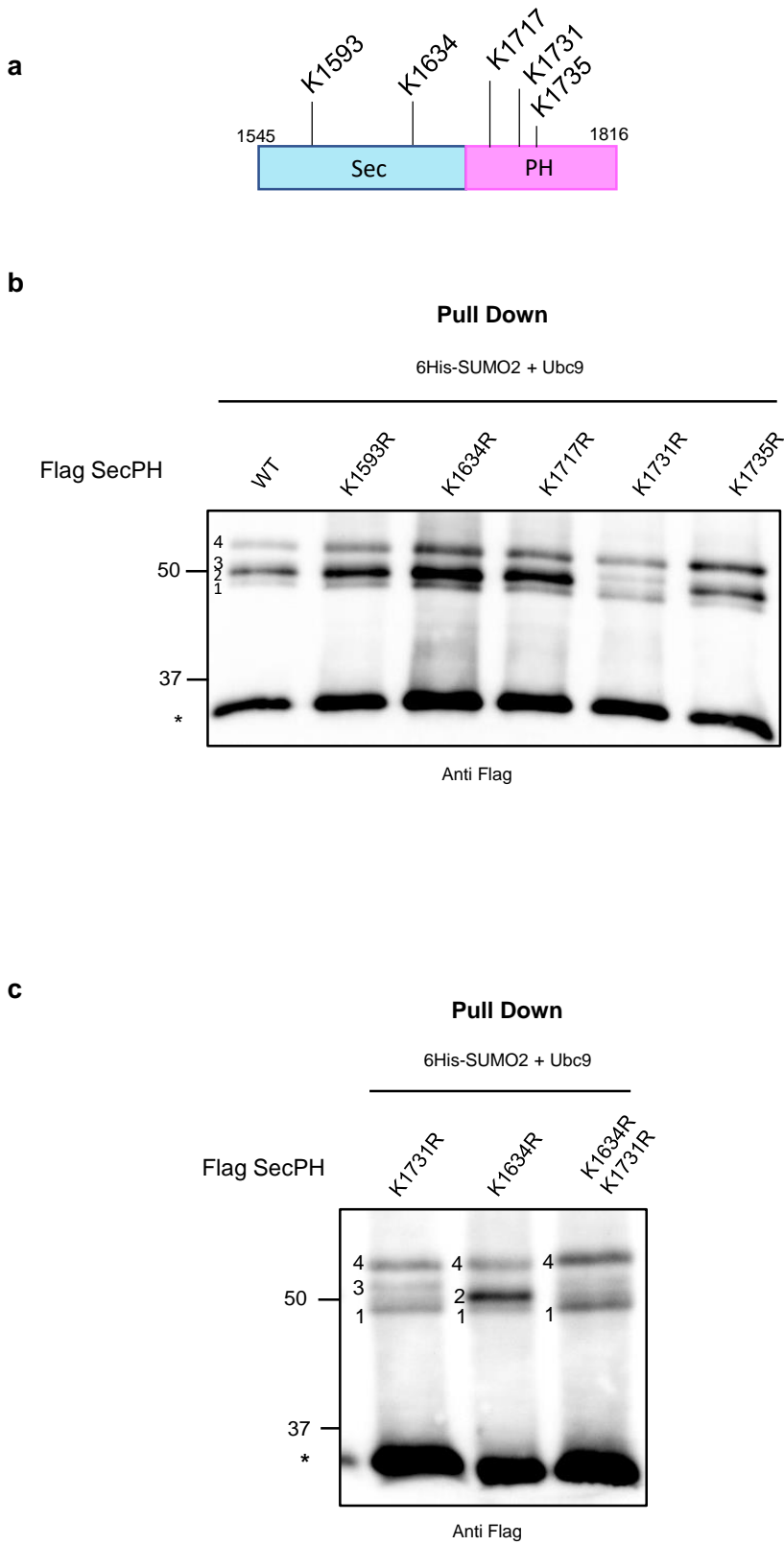
**Figure 1. Localization of putative SUMO acceptor lysines in Nf1 domains and detection of different forms of SUMOylation of the Nf1 SecPH domain.**

**a.** Localization of putative SUMO acceptor lysines in Nf1 domains of Neurofibromin isoform 2 (2818 amino acids, NP-000258.1). NTD (N-terminal Domain) in navy blue, GRD (GAP-related domain) in teal, Sec in sky blue, PH (Pleckstrin Homology) in magenta, CDM (Central Dimerisation Module) in green and CTD (C-terminal Domain) in purple. The black bars indicate the interdomain linkers. Nf1 was analysed with SUMO PLOT and JASSA bioinformatic tools. Lysines predicted to have a high probability of SUMOylation by both software programs are indicated in bold letters and underlined (**K1717**, **K2006**). Lysines predicted to have a high probability of SUMOylation by one software and a low probability by the other are indicated in bold (**K24**, **K428**, **K783**, **K798**, **K1634**, **K1866**). The other lysines were predicted to be SUMOylated with a high probability by only one software (K1531, K1593, K1731, K1735, K2259).

**b.** Enrichment in SUMOylated proteins of HEK293 cells transfected with different plasmids. Cells were co-transfected with p3X Flag SecPH, pcDNA3 6His-SUMO2, and pcDNA3 Ubc9 or only p3X Flag SecPH as a negative control. After 24 h of growth, cells were lysed in 6M guanidinium buffer and SUMO2/3 and SUMOylated proteins were enriched by pull down using cobalt beads. Blotting of the eluates with anti SUMO2/3 antibodies indicates that SUMO2/3 are integrated in various cellular proteins whereas the 6His-SUMO2 free protein migrates at a molecular mass of 15 kDa.

**c.** SUMOylation of Flag SecPH in the eluted samples isolated in **(b)** is detected with anti Flag antibodies. Four bands migrating around 50 kDa and denoted 1 to 4, correspond to different monoSUMOylated Flag SecPH species and the lower band around 37 kDa (marked by \*) represents the unSUMOylated Flag SecPH non specifically bound to the cobalt beads. Note that the detection of overproduced nonSUMOylated proteins in SUMO-enriched fractions is a frequently observed issue with such approaches.

Figure 2



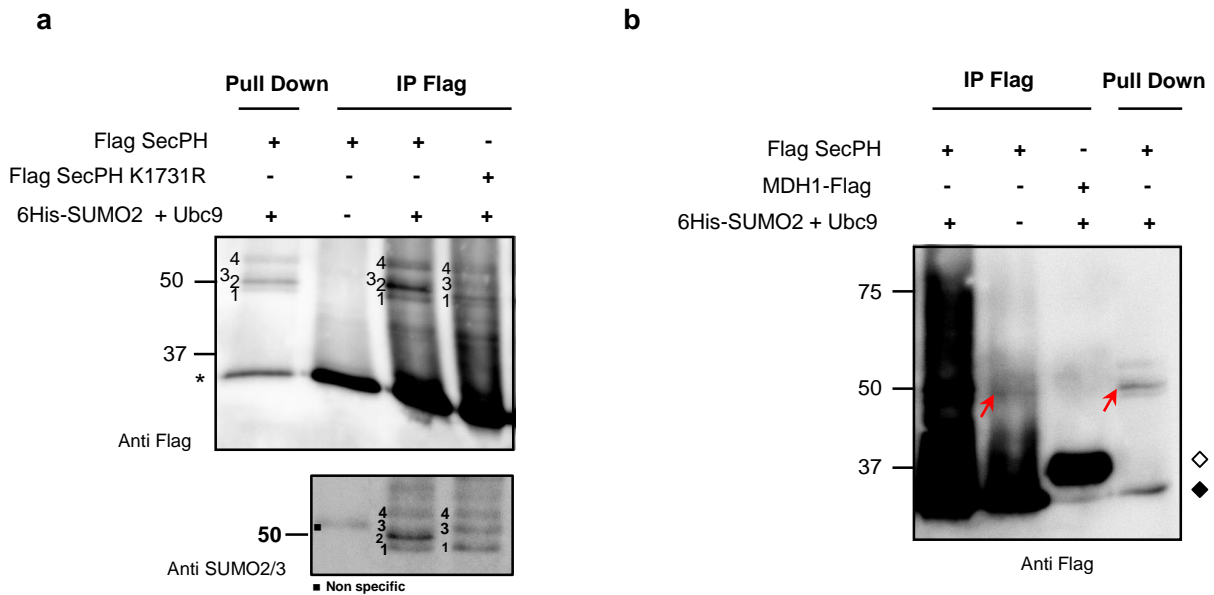
**Figure 2. K1731 and K1634 contribute to SecPH SUMOylation profile, as revealed by Pull Down analysis**

**a.** Schematic representation of Sec and PH domains of human Nf1 (in blue and pink, respectively) and the position of the five bioinformatically-predicted putative SUMOylated lysines.

**b.** The five putative SUMOylated lysines were individually substituted with arginine in p3X Flag SecPH (p3X Flag SecPH K1593R, p3X Flag SecPH K1634R, p3X Flag SecPH K1717R, p3X Flag SecPH K1731R, and p3X Flag SecPH K1735R) to identify the SUMOylated SecPH species in these mutants after SUMOylated proteins enrichment as described in Fig. 1**b**. The four bands denoted 1 to 4 highlight the SecPH SUMOylated species and unSUMOylated Flag SecPH derivatives bound nonspecifically to cobalt beads are marked with an asterisk.

**c.** The p3X Flag SecPH derivatives: p3X Flag SecPH K1634R, p3X Flag SecPH K1731R, and p3X Flag SecPH K1634R K1731R, were co transfected and analysed as in Fig. 1**b,c**. SUMOylated SecPH species are shown (bands 1, 2, 3, and 4). The nonspecific binding of unSUMOylated Flag SecPH derivatives to the cobalt beads is indicated by an asterisk.

**Figure 3**



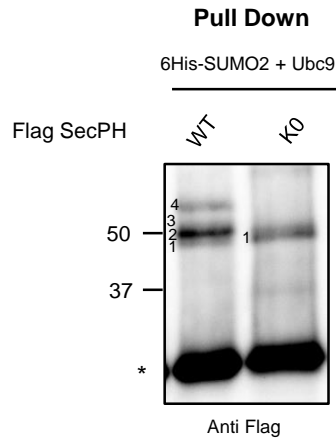
**Figure 3. K1731 is involved in SecPH SUMOylation, as revealed by Flag Immunoprecipitation**

**a.** SUMO-modified Flag SecPH and Flag SecPH K1731R derivative were analysed after Flag immunoprecipitation of HEK293 cells co-transfected with plasmids encoding these proteins and pcDNA3 Ubc9 and pcDNA3 6His-SUMO2 (or empty vectors as controls). The immunoblots revealed with anti Flag antibodies (upper panel) are presented along with the eluates from the pull down experiments presented in Fig. 1c. In the lower panel, the immunoprecipitated samples are revealed with anti SUMO2/3 antibodies. On the two blots, the four SecPH monoSUMOylated species in the Flag SecPH lane are labelled 1, 2, 3 and 4. The asterisk in the upper panel indicates nonspecific binding of unSUMOylated Flag SecPH to the cobalt beads for the pull down lane, and immunoprecipitated unSUMOylated Flag SecPH and Flag SecPH K1731R derivative for the IP Flag lanes. The square indicates a non specific band revealed by the anti SUMO2/3 antibodies (lower panel).

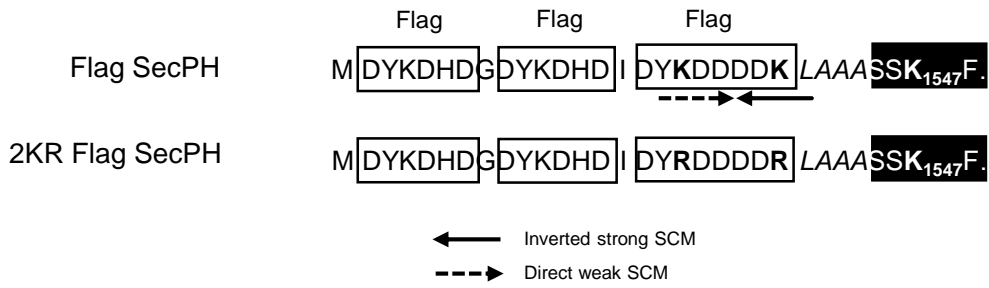
**b.** To evaluate K1731 SUMOylation under endogenous SUMO levels, SUMO-modified SecPH (expressed from p3X Flag SecPH) was analyzed after Flag immunoprecipitation of lysates of HEK293 cells co-transfected with or without SUMO2 and Ubc9 overexpression plasmids; MDH1-Flag is a negative control. An over-exposure of the immunoblot revealed with anti Flag antibodies is shown along with the eluates from the pull down experiment presented in Fig. 2b (the pull down sample was loaded in less amount as compared to usual in order to avoid signal saturation). A band migrating as band 2 in Fig. 3a is indicated by a red arrow. The unmodified immunoprecipitated Flag SecPH and Flag SecPH K1731R derivative is indicated with a filled diamond and the unmodified immunoprecipitated MDH1-Flag is shown with an empty diamond.

Figure 4

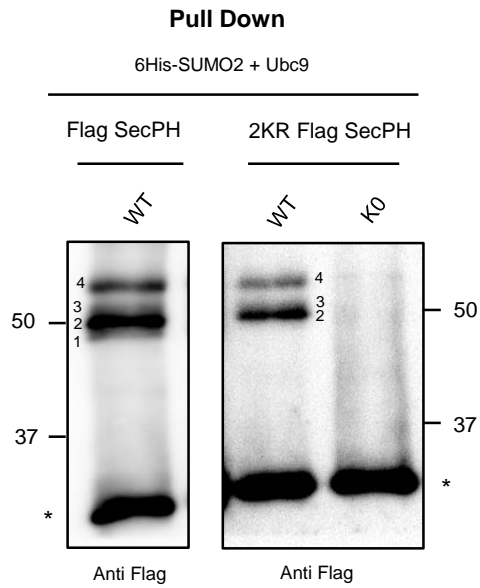
a



b



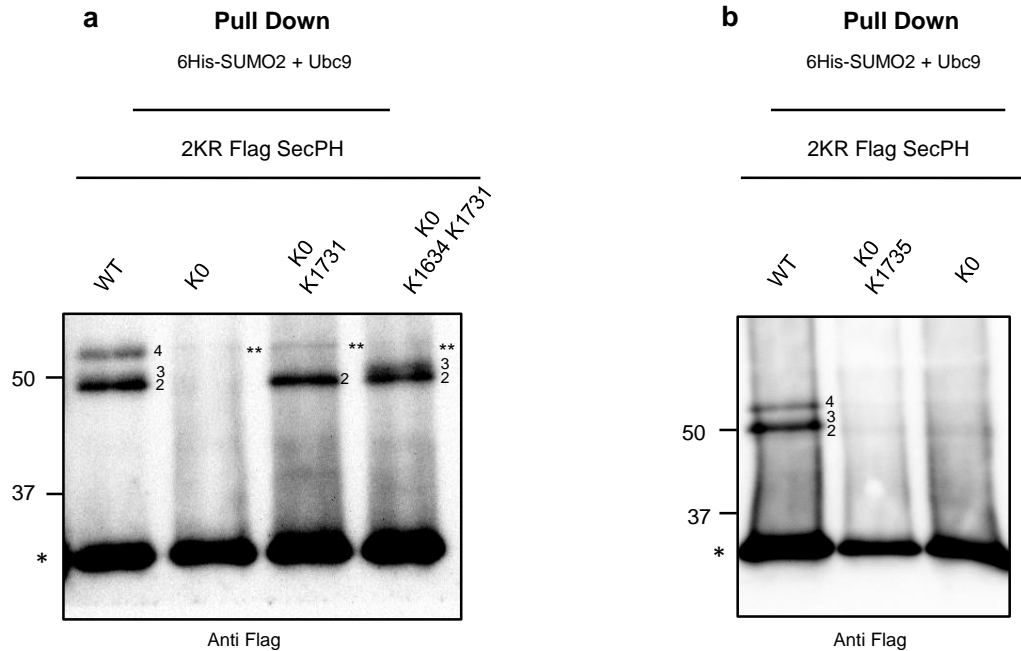
c



#### Figure 4. The Flag Tag is SUMOylated

- a.** Enrichment in SUMOylated proteins of HEK293 co-transfected with p3X Flag SecPH K0, a SecPH derivative with the 21 lysines in SecPH replaced by arginines, or the wild type control, was assessed by western blot with anti Flag antibodies as in Fig. 1c. In p3X Flag SecPH K0, bands 2, 3, and 4 are lost whereas a large SUMOylation band migrating at the same position as band 1 (in Fig. 1c) is revealed. The asterisk (\*) indicates nonspecific binding of unSUMOylated Flag SecPH and Flag SecPH K0 to cobalt beads.
- b.** Partial amino acid sequence of Flag SecPH and 2KR Flag SecPH. The three Flag sequences are boxed with the two lysines of the last Flag in bold, the linker is in italics, and the N-terminus of SecPH fused in frame is represented by a black box. The direct and inverted SUMOylation consensus motifs (SCM) are highlighted by a dashed arrow and a solid arrow, respectively. In 2KR Flag SecPH, the two lysines of the last Flag were replaced by arginine (in bold).
- c.** Enrichment in SUMOylated proteins of HEK293 co-transfected with p3X Flag SecPH, and plasmids encoding 2KR Flag SecPH WT and 2KR Flag SecPH K0 was assessed as in (a). In the left panel, the 4 bands corresponding to monoSUMOylated SecPH species are indicated. In the right panel, a loss of band 1 is observed in both lanes whereas bands 2, 3 and 4 are maintained in the 2KR Flag SecPH WT lane, and lost in the 2KR Flag SecPH K0 lane. The asterisk (\*) indicates nonspecific binding of unSUMOylated Flag SecPH derivatives to the cobalt beads.

**Figure 5**



**Figure 5. K1634 and K1731 constitute minor and major SUMO-conjugation sites in SecPH**

**a.** Enrichment in SUMOylated proteins of HEK293 co-transfected with plasmids encoding 2KR Flag SecPH WT, 2KR Flag SecPH K0, 2KR Flag SecPH K0 in which K1731 was reintroduced and 2KR Flag SecPH K0 in which K1634 and K1731 were reintroduced.

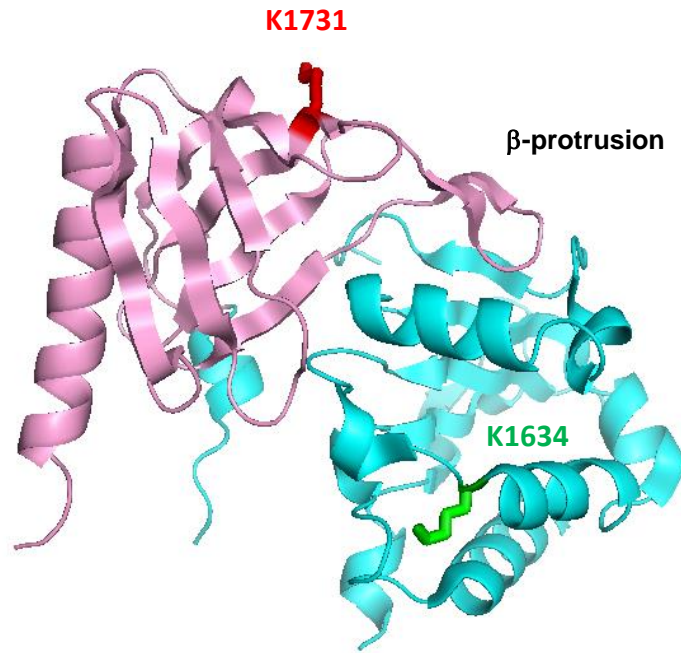
In 2KR Flag SecPH WT, bands 2, 3 and 4 are observed and band 1 which was seen in Flag SecPH disappears (Fig. 3c) whereas in 2KR Flag SecPH K0, all four bands disappear. Band 2 is recovered when K1731 is reintroduced in 2KR Flag SecPH K0, bands 2 and 3 are recovered when K1634 and K1731 are reintroduced in 2KR Flag SecPH K0. Of note, the double asterisk (\*\*) indicates a nonspecific band migrating slightly above band 4. The asterisk (\*) indicates nonspecific binding of unSUMOylated 2KR Flag SecPH derivatives to the cobalt beads.

**b.** K1735 is not SUMOylated when reintroduced into a 2KR Flag SecPH K0 mutant. Enrichment in SUMOylated proteins of HEK293 cell co-transfected with plasmids encoding 2KR Flag SecPH WT, 2KR Flag SecPH K0 and 2KR Flag SecPH K0 K1735 (a SecPH K0 in which K1735 was reintroduced). The immunoblot revealed with anti Flag antibodies shows the loss of bands 2, 3 and 4 in both lanes K0 K1735 and K0. The nonspecific binding of unSUMOylated 2KR Flag SecPH derivatives to the cobalt beads is indicated by an asterisk.



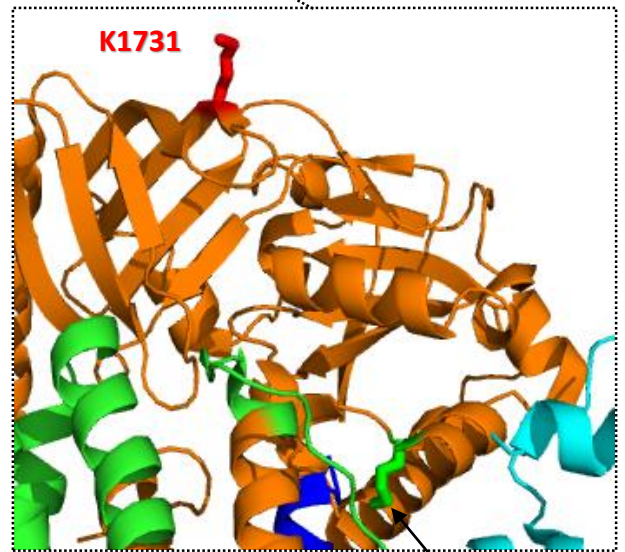
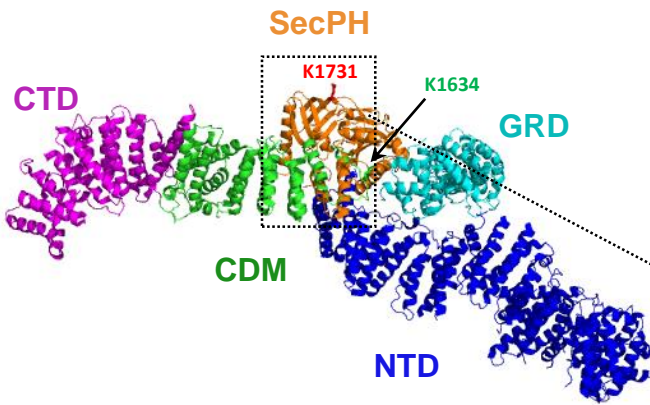
Figure 6

a

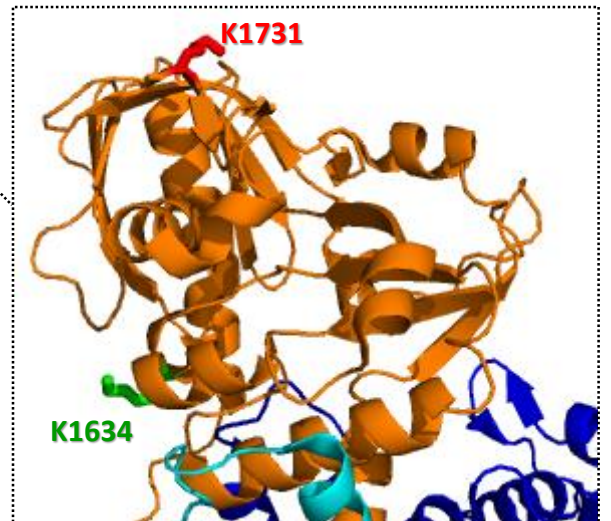
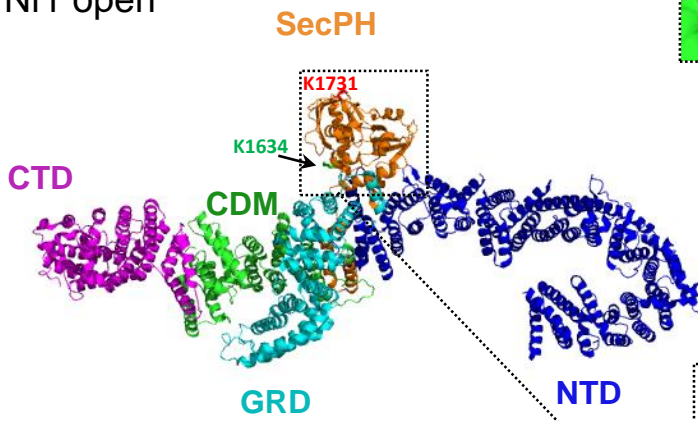


b

Nf1 closed



Nf1 open

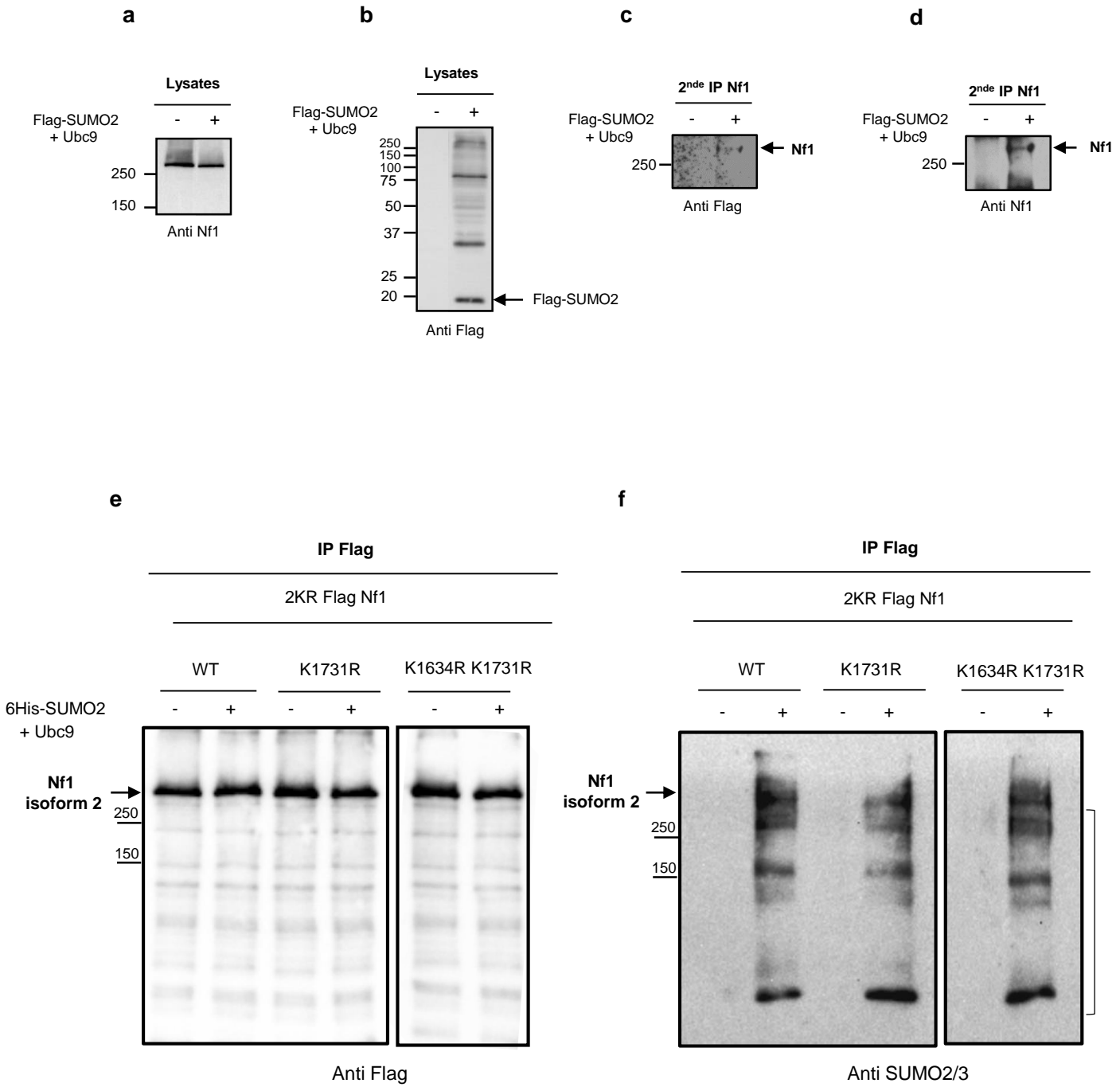


**Figure 6. Positions of K1634 and K1731 SUMO-conjugated lysines in the 3D structure of SecPH (a) and in Nf1 isoform 2 (NP-000258.1) 3D-structure (b)**

**a.** Ribbon representation of the Nf1-SecPH 3D structure with Sec in light blue and PH in pink (PDB ID: 2E2X). K1634 and K1731 side chains are represented by sticks and labelled in green and red, respectively.

**b.** Overall ribbon representation of Nf1 isoform 2 3D structure in closed (PDB ID: 7R03) (upper panel) and in open (PDB ID: 7R04) (lower panel) conformations. Nf1 domains as defined by Chaker-Margot et al., 2022 (Ref. 26) are colored differently: NTD (N-terminal Domain) in navy blue, GRD in cyan, SecPH in orange, CDM (Central Dimerisation Module) in green and CTD (C-terminal Domain) in magenta. Fragments zoomed-in are marked with dotted squares.

**Figure 7**



**Figure 7. Endogenous Nf1s? and exogenously produced Nf1 isoform 2 and derivatives are SUMOylated by SUMO2**

**a.** Total lysates prepared from HEK293 cells co-transfected with p3X Flag SUMO2, along with pcDNA3 Ubc9 (or the corresponding empty plasmids for the negative control) were analysed by western-blot with anti Nf1 antibodies to show similar protein levels in the lysates.

**b.** The same lysates as in (a) analysed with anti Flag antibodies show that Flag-SUMO2 is integrated into different SUMOylated proteins (Flag-SUMO2 migration is different from Fig.1b where the SUMO2 is tagged with 6His. The Flag tag (3xFlag) increases its molecular weight to 18 kDa, arrow).

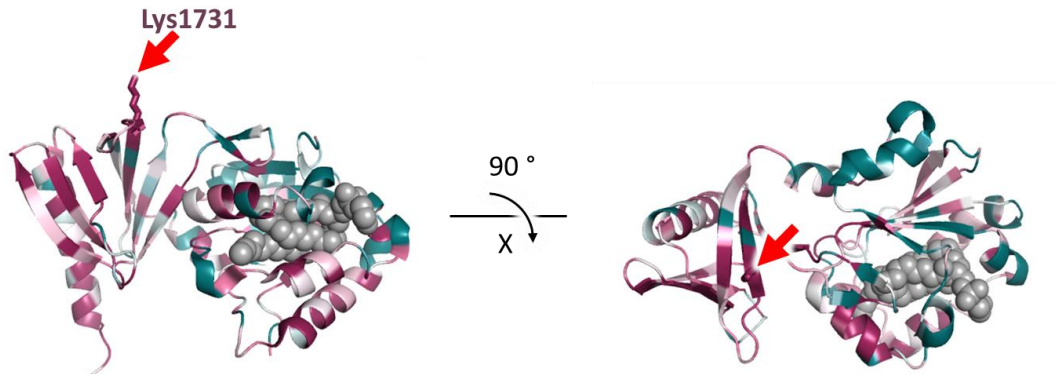
**c, d.** Total lysates were subjected to an initial immunoprecipitation (IP) with anti Flag antibodies for the enrichment of SUMOylated proteins or SUMO-interacting proteins, followed by a second IP with anti Nf1 antibodies. Western blot analyses of the eluates of the second IP shown with anti Flag antibodies (c) and anti Nf1 antibodies (d) demonstrate that endogenous Nf1s is are SUMOylated by SUMO2.

**e, f.** HEK293 cells were co-transfected with p3X 2KR Flag Nf1 isoform 2 or derivative plasmids encoding the corresponding K1731R or K1731R K1634R mutants (unSUMOylable Flag tag in p3X 2KR Flag SecPH), pcDNA3 6His-SUMO2 and pcDNA3 Ubc9 plasmids (these two later plasmids were replaced by empty plasmids for the negative control) and total lysates were subjected to an IP Flag. Eluates revealed with anti Flag antibodies (e) and anti SUMO2/3 antibodies (f) demonstrate that exogenously produced 2KR Flag Nf1 isoform 2, K1731R and K1731R K1634R mutants are SUMOylated by SUMO2. The bracket in (f) indicates SUMO-modified proteins co-immunoprecipitated with Nf1.

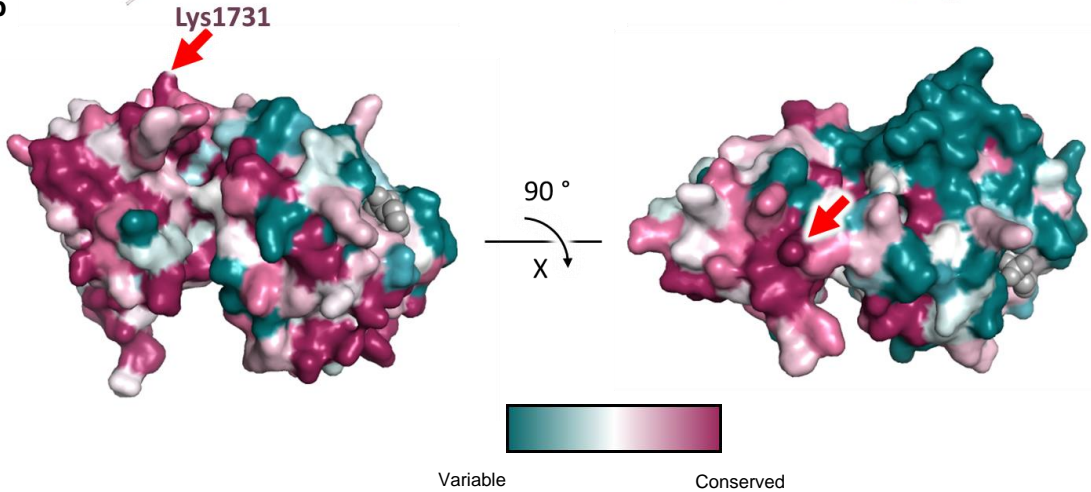
Figure 8

## K1731 is highly conserved

a



b



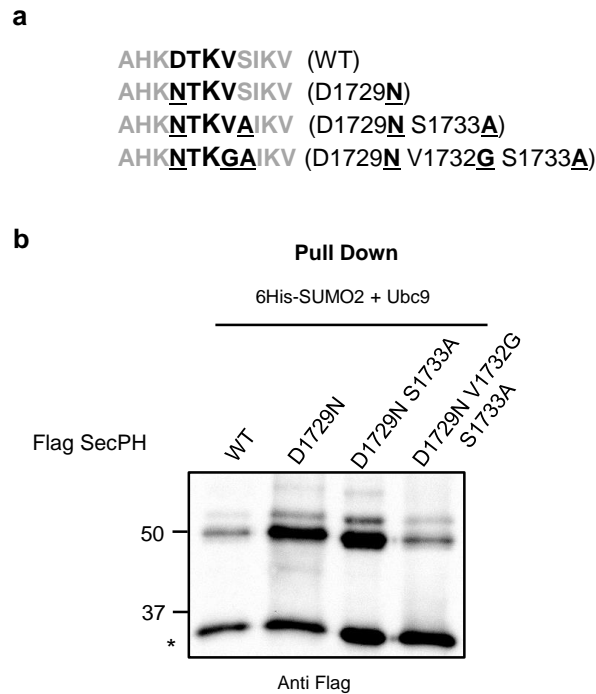
**Figure 8. K1731 is highly conserved in most metazoan Nf1 orthologues**

Sequence conservation analysis was performed using the ConSurf 2016 web server with the SecPH crystal structure in complex with PtdEtn (PDB ID: 2E2X). PtdEtn is shown as grey spheres.

**a.** SecPH is shown in ribbon representation in the same orientation as in Fig. 6a and after the indicated rotation. The side chain of the main SUMOylation site, Lys1731, is shown as sticks and indicated with a red arrow.

**b.** The same views as in (a) but in surface representation. The main SUMOylation site, Lys1731, is indicated with a red arrow.

**Figure 9**



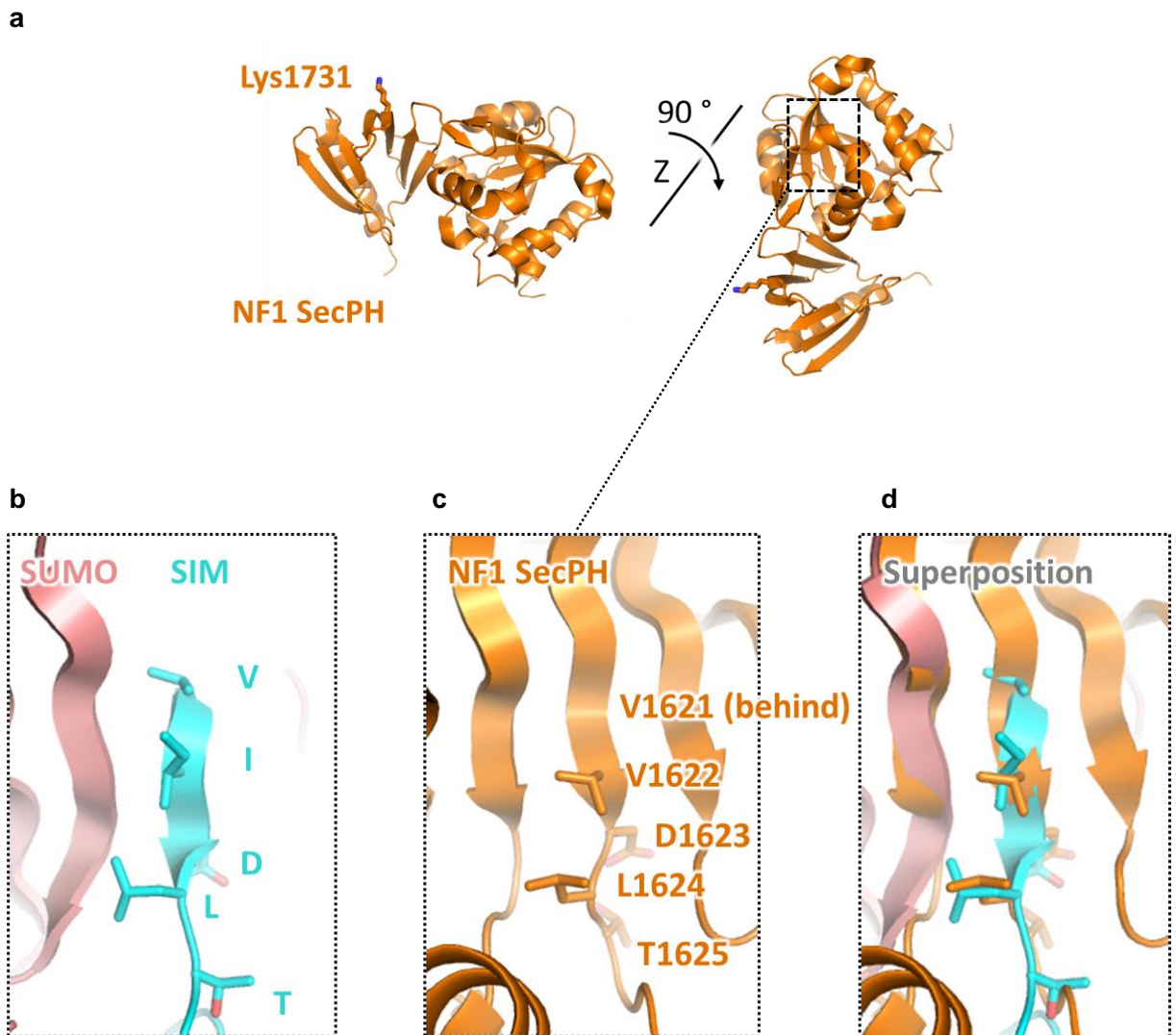
**Figure 9. The SUMO consensus motif is dispensable for the SUMOylation of SecPH on K1731**

**a.** The sequence of the “predicted” inverted SUMOylation consensus motif (DTK<sub>1731</sub>V) is written in black and bold and the amino acids surrounding it in grey; the SUMO acceptor site, K1731, is shown by the increased font size. The conserved amino acid residue D1729 was replaced by N, the potentially phosphorylated S1733 mutated to A was then introduced into the D1729N mutant, and finally, a triple mutant that completely abolished the consensus motif was created by changing the conserved hydrophobic residue V1732 to G in D1729N S1733A.

**b.** Enrichment in SUMOylated proteins of HEK293 cells co-transfected with p3X Flag SecPH or p3X Flag SecPH derivatives harbouring the indicated mutations in (a). The immunoblot revealed with anti Flag antibodies shows an identical SUMOylation profile in Flag SecPH WT and Flag SecPH derivative mutants. The asterisk (\*) indicates nonspecific binding of unmodified Flag SecPH derivatives to the cobalt beads.



**Figure 10**



**Figure 10. The predicted SecPH SIM domain which could be involved in K1731 SUMOylation is not accessible**

**a.** SecPH (PDB ID: 2E2X) is shown in the same orientation as in Fig. 6a and after the indicated rotation. A fragment zoomed-in in (c) is marked with a dashed square. The side chain of the main SUMOylation site, Lys1731, is shown as sticks.

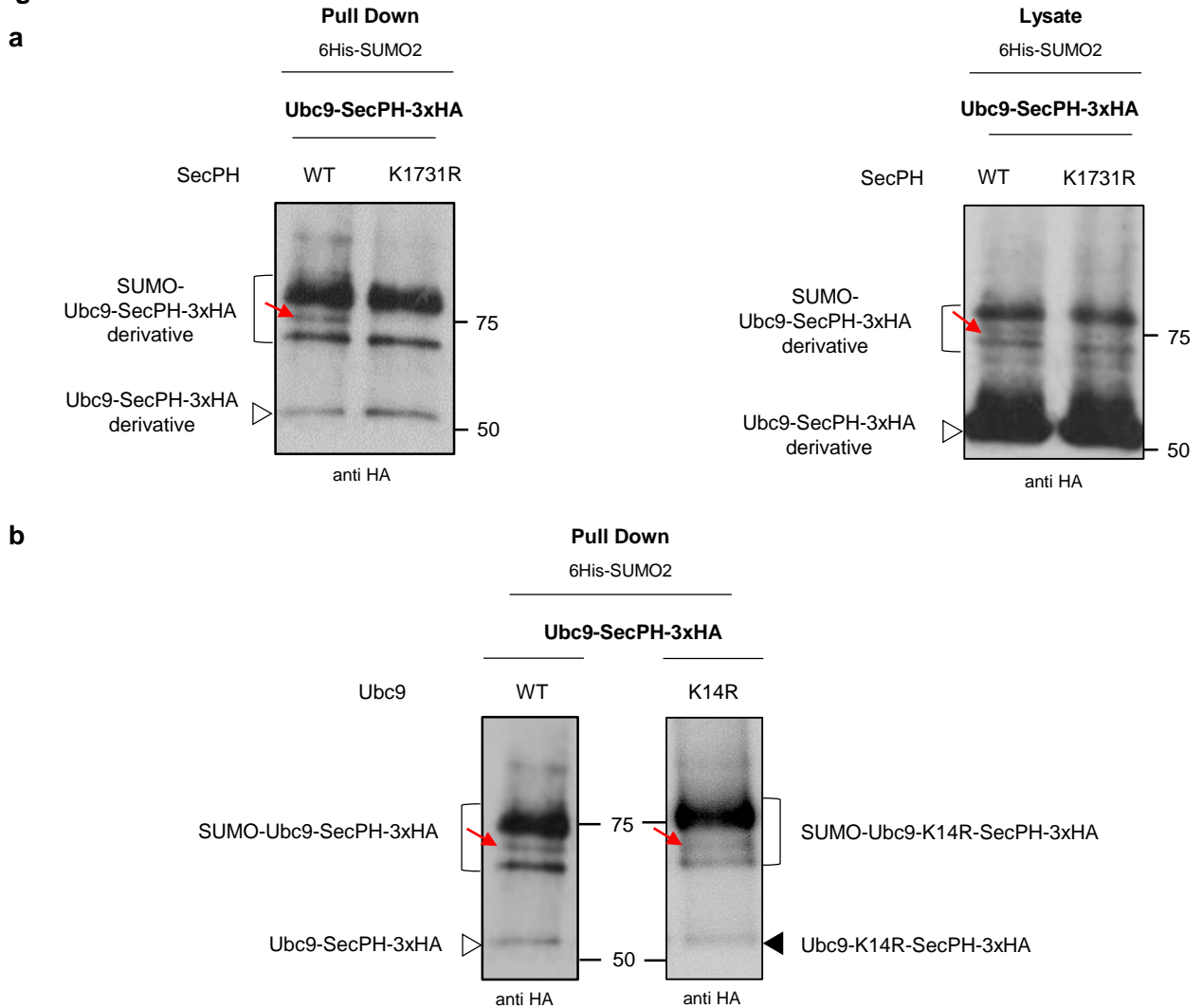
**b.** The molecular interface between the SIM<sup>CAF1</sup> and SUMO3 (PDB ID: 2RPQ). SUMO is shown in ribbon representation in pink and SIM<sup>CAF1</sup> is shown in stick representation in blue.

**c.** A fragment of the Nf1 SecPH domain 3D structure surrounding its predicted SIM motif (VVDLT) located in the central  $\beta$ -sheet of the Sec domain. Residues corresponding to this predicted SIM are labelled and their side chains are shown.

**d.** The superposition of (b) and (c) demonstrates that the predicted SIM domain in SecPH, due to being located on the central  $\beta$ -strand of a  $\beta$ -sheet, is unavailable for binding to SUMO.



**Figure 11**

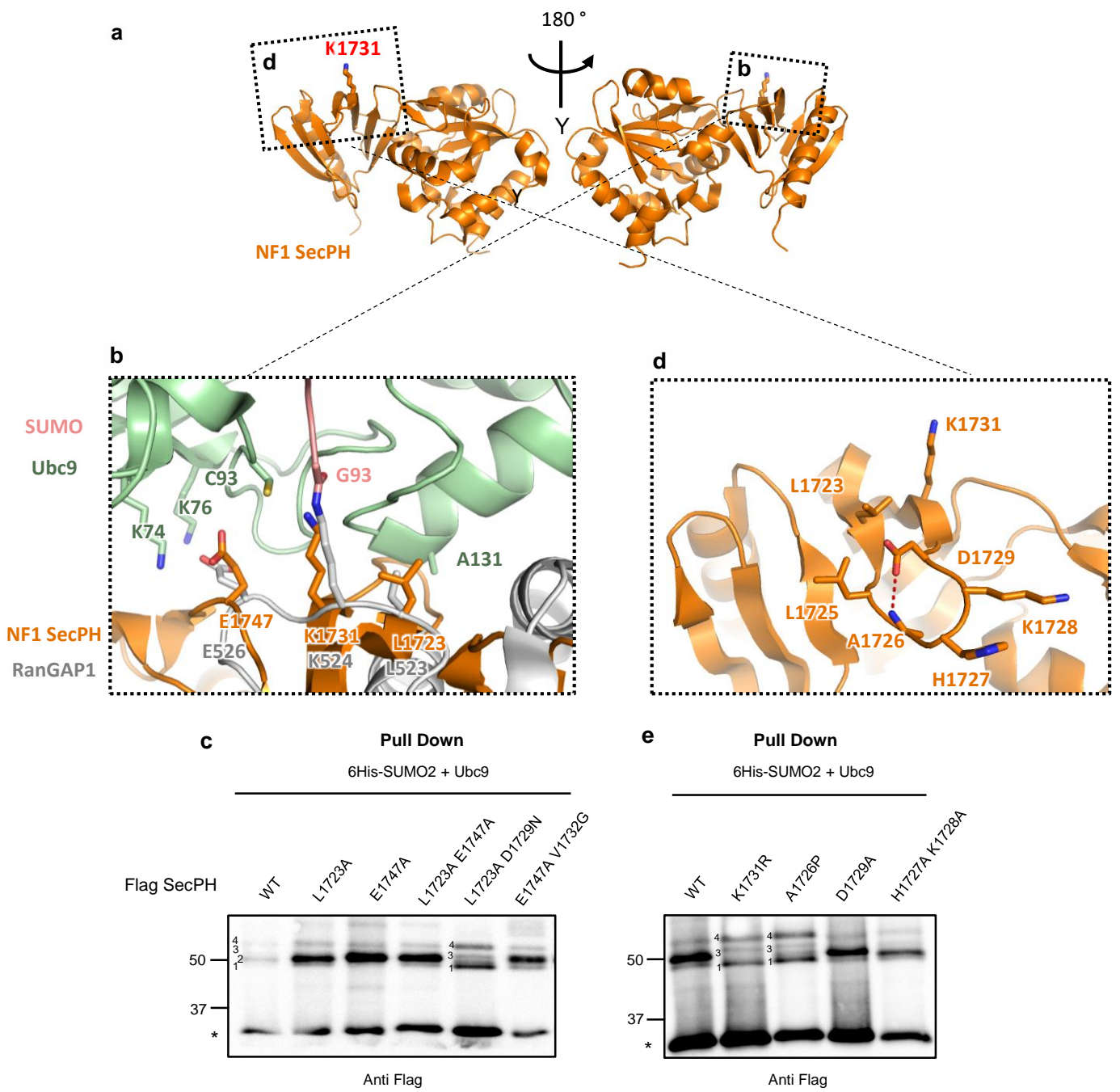


**Figure 11. Prevention of Ubc9 auto-SUMOylation in Ubc9-SecPH-3xHA fusion protein does not interfere with K1731 SUMOylation**

**a.** HEK293 cells were co-transfected with pcDNA3 6His-SUMO2 along with a plasmid encoding Ubc9-SecPH-3xHA fusion protein, or a SecPH K1731R derivative. After 24 h of growth, cells were lysed in 6M guanidinium buffer and SUMOylated proteins were enriched by pull down using cobalt beads. Blotting of the eluates with anti HA antibodies is shown on the left panel. In the right panel, the proteins in the lysates detected with anti HA antibodies are shown. The SUMOylated Ubc9-SecPH-3xHA and Ubc9-SecPH K1731R-3xHA fusion proteins are indicated with a bracket. Red arrow highlights the SUMOylated K1731 residue. The unSUMOylated Ubc9-SecPH-3xHA and Ubc9-SecPH K1731R-3xHA fusion proteins are indicated with a triangle (54 kDa) on both panels.

**b.** Same experiment as in (a) with cells co-transfected with pcDNA3 6His-SUMO2 and pUbc9-SecPH-3xHA (left panel) or pUbc9-K14R-SecPH-3xHA (right panel). The bracket indicates the SUMOylated Ubc9-SecPH-3xHA (left) and Ubc9-K14R-SecPH-3xHA (right) fusion proteins. Red arrow highlights the SUMOylated K1731 residue. The empty and filled triangles indicate the unSUMOylated Ubc9-SecPH-3xHA and Ubc9-K14R-SecPH-3xHA fusion proteins respectively (54 kDa).

Figure 12



**Figure 12. Loop 1723-1729 on SecPH contributes to the recognition of K1731 for SUMOylation**

**a.** Nf1 SecPH (PDB ID: 2E2X) is shown in the same orientation as in Fig. 6a and after the indicated rotation. Fragments zoomed-in in panels **b** and **d** are marked with dotted squares. The side chain of the main SUMOylation site, Lys1731, is shown as sticks.

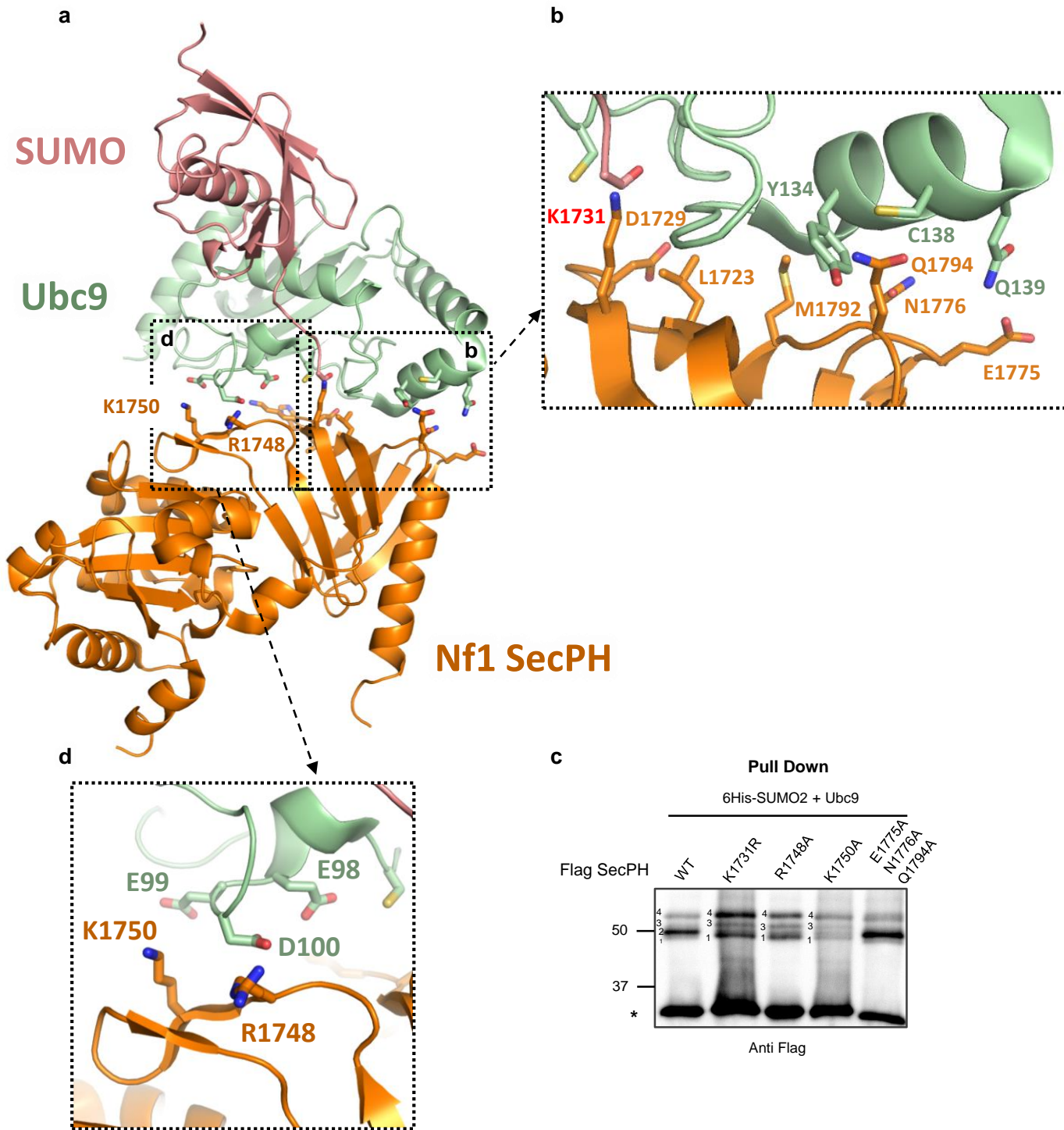
**b.** Nf1 SecPH interaction with Ubc9 was modelled from the structure of a complex comprising RanGAP1, SUMO, Ubc9 and an E3 SUMOylation ligase (PDB ID: 5D2M, Ref 62). A ribbon representation is shown with RanGAP1, SUMO, Ubc9, and Nf1 SecPH coloured in grey, pink, green and orange, respectively. The side chains of residues potentially involved in K1731 SUMOylation are shown as sticks.

**c.** Substitutions of amino acids belonging to the putative “3D SCM”, alone or in combination with substitutions of amino acid residues of the potential inverted SCM were introduced into p3XFlag SecPH and the resulting plasmids transfected into HEK293 cells, along with pcDNA3 6His-SUMO2 and pcDNA3 Ubc9. Cell lysates were subjected to pull down analysis as described in Fig. 1c. The immunoblots revealed with anti Flag are shown.

**d.** Close-up view of the short surface loop surrounding K1731 located between residues L1723 and D1729. The red dotted line indicates a hydrogen bond involving the peptide amide between L1725 and A1726 and the carbonyl of D1729. Residues potentially important are labelled.

**e.** Substitutions of different amino acid of the L1723-D1729 loop were introduced into p3XFlag SecPH and processed as in (c). The asterisk in (c) and (e) indicates nonspecific binding of unSUMOylated Flag SecPH derivatives to the cobalt beads.

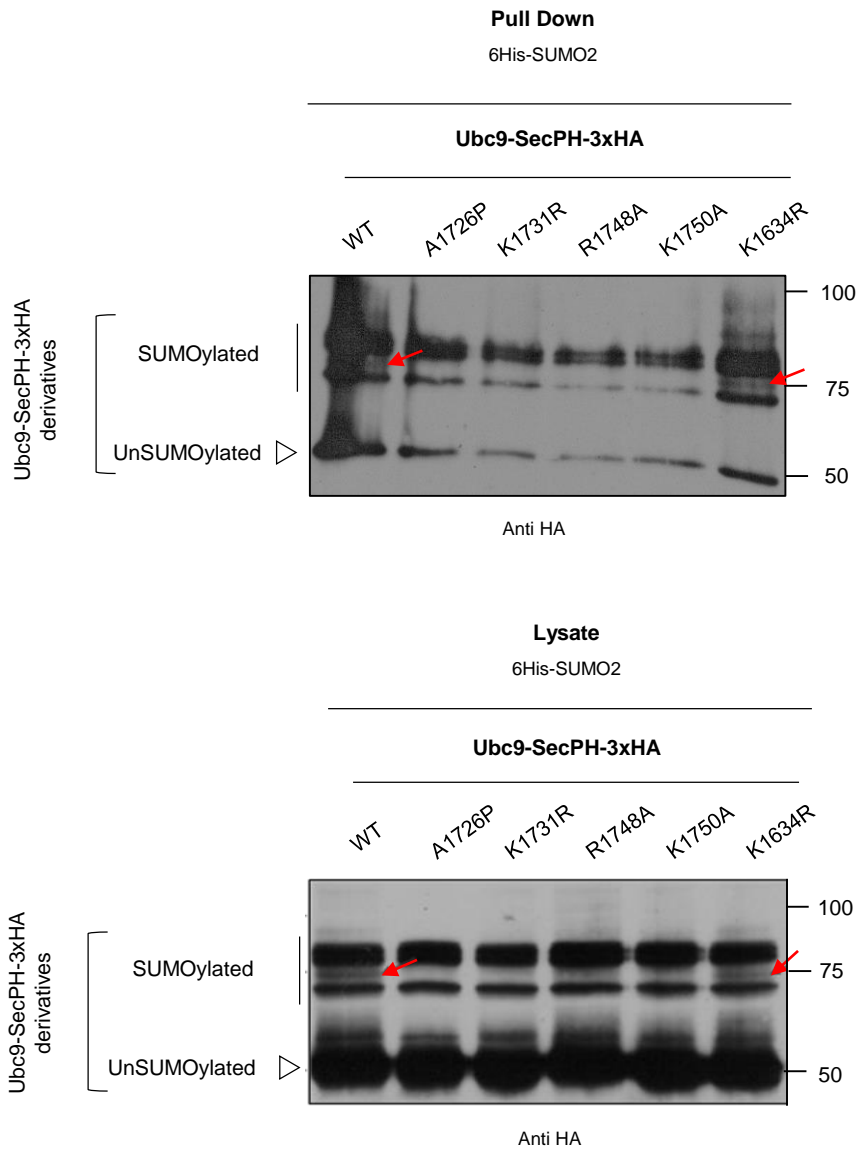
Figure 13



**Figure 13. Positively charged residues on SecPH  $\beta$ -protrusion are essential for SecPH SUMOylation on K1731**

- a.** Overall view of the model of the interaction of Nf1 SecPH with Ubc9 and SUMO as described in Fig. 12b with Nf1SecPH in orange, SUMO in pink, and Ubc9 in green. Fragments zoomed-in in (b) and (d) are framed.
- b.** Close-up view of the two surface loops identified (delineated by residues E1775 to N1777, and M1792 to E1795).
- c.** The triple mutant E1775A, N1776A, Q1794A was made in p3X Flag SecPH, co-transfected in HEK293 along with pcDNA3 6His-SUMO2 and pcDNA Ubc9 and processed as in Fig. 1c. The immunoblots revealed with anti Flag are shown. The asterisk indicates nonspecific binding of unSUMOylated Flag SecPH derivatives to the cobalt beads.
- d.** Close-up view of the  $\beta$ -protrusion whose positively charged residues R1748 and K1750 could interact with the negatively charged residues of Ubc9 (E98, E99, D100) for efficient positioning of Ubc9. These residues were individually substituted by Alanine in p3X Flag SecPH and analysis of K1731 SUMOylation conducted as in Fig. 1c is shown in (c).

**Figure 14**

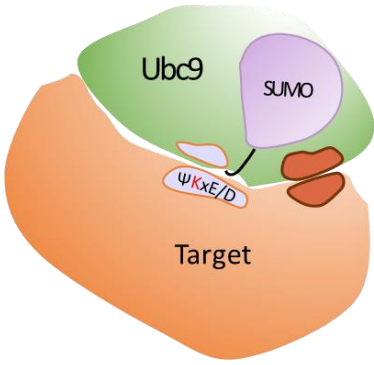


**Figure 14. K1731 SUMOylation band disappears in mutants located into the structural elements required for SecPH SUMOylation (A1726P, R1748A and K1750A)**

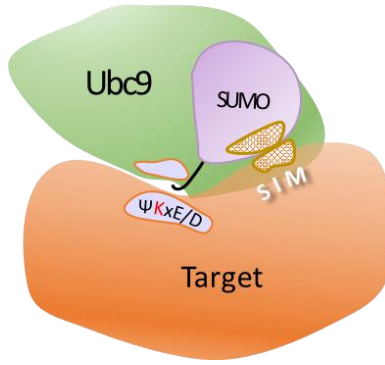
HEK293 cells were co-transfected with pcDNA3 6His-SUMO2 along with a plasmid encoding Ubc9-SecPH-3xHA fusion protein, or SecPH Ubc9-SecPH-3xHA fusion protein derivatives K1634R, A1726P, K1731R, R1748A, K1750A. After 24 h of growth, cells were lysed in 6M guanidinium buffer and SUMOylated proteins were enriched by pull down using cobalt beads. Blotting of the eluates and lysates with anti HA antibodies is shown on the top and bottom panels, respectively. The vertical bar shows the SUMOylated Ubc9-SecPH-3xHA fusion protein derivatives. Red arrow highlights the SUMOylated K1731 residue. UnSUMOylated Ubc9-SecPH-3xHA fusion protein derivatives are indicated with a triangle (54 kDa).

Figure 15

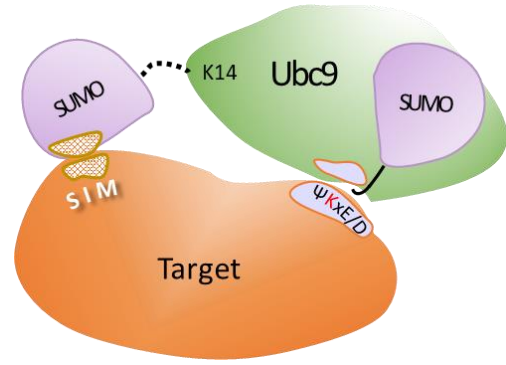
a Extended SCM sequence



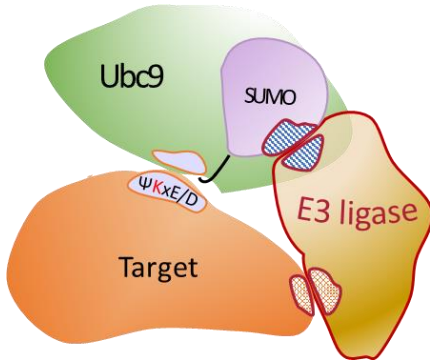
b SIM close to SCM



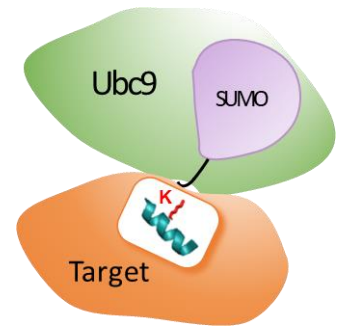
c Ubc9 SUMOylation on K14



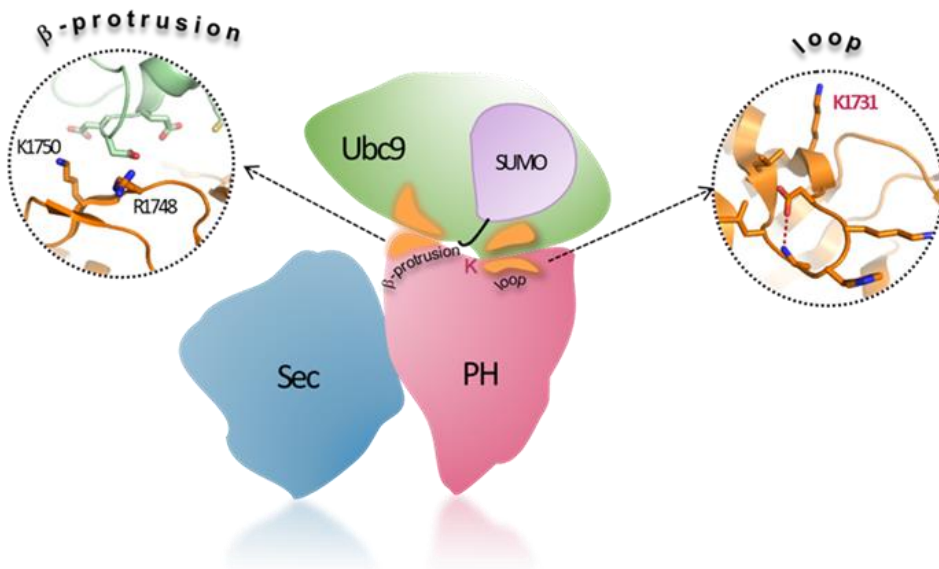
d E3 ligase



e α-helix



f Nf1 SecPH





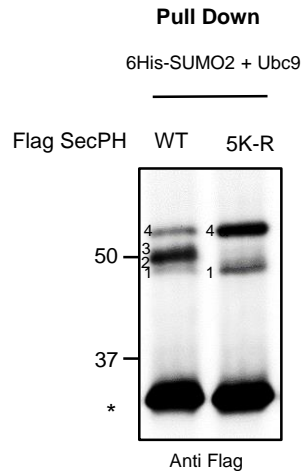
**Figure 15. Models illustrating different mechanisms governing SUMOylation site selection**

Various mechanisms of SUMOylation site selection are shown in different cartoons. Ubc9 is represented in green, SUMO in pink and the target (except SecPH) in brown. Ubc9 is linked to SUMO by a thioester bond at C93 (plain hook),  $\psi$ KxE/D indicates the SCM.

- a.** Site selection involving the target SCM and an extended SCM sequence (e.g. RanGAP1). The interaction interfaces are indicated on the target and Ubc9.
- b.** Site selection involving the target SCM and a target SIM which interacts with the SUMO linked to Ubc9 (e.g. PML). The interaction interfaces are indicated on the target, Ubc9 and SUMO.
- c.** Site selection involving the target SCM, a target SIM and Ubc9 conjugated to SUMO on K14 (indicated by a dashed hook), (e.g. Sp100). The interaction interfaces are indicated on the target, Ubc9 and SUMO conjugated to Ubc9.
- d.** Site selection involving the target SCM and a E3 ligase (PCNA K164). The interaction interfaces are indicated on the target, Ubc9, SUMO and E3 ligase.
- e.** Site selection involving a secondary structural element ( $\alpha$ -helix) of the target (E2-25K or Ubc9). The helical motif is in teal and the modified K (K14) and its side chain (stick representation) are labelled in red.
- f.** K1731 selection in SecPH. In this cartoon, Sec is indicated in blue and PH in purple. The two interaction interfaces on PH and Ubc9 are indicated along with K1731 (as a red K). SecPH surface-exposed structural elements ( $\beta$ -protrusion and loop) involved in K1731 selection are zoomed-in in ribbon representation in two close-up views marked with dotted circles.



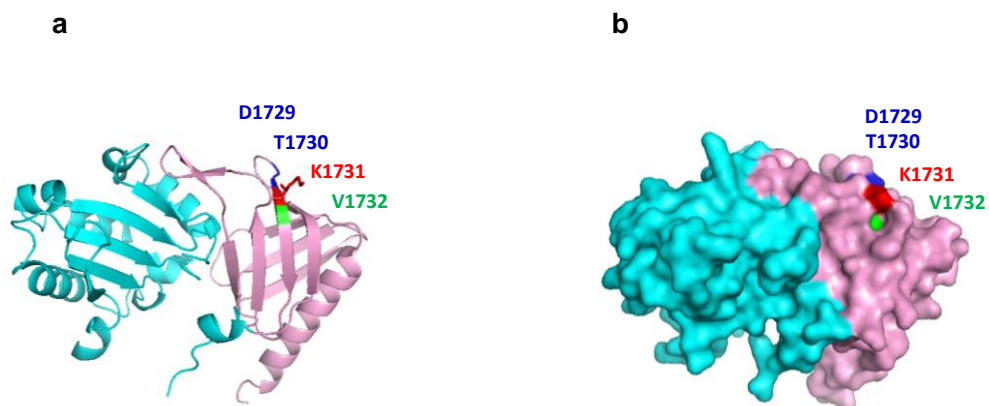
## Supplementary Figure 1



### Figure S1. The three other putative acceptor lysines of SecPH are not SUMOylated

The potential SUMOylation of the three other SecPH SUMOylation consensus motifs was checked by enrichment in SUMOylated proteins of HEK293 cells co-transfected with pcDNA3 6His-SUMO2 and the p3X Flag SecPH control plasmid or a SecPH plasmid derivative in which the five putative SUMOylated lysines in SecPH have been replaced by arginine: p3X Flag SecPH 5K-R (p3X Flag SecPH K1593R, K1634R, K1717R, K1731R, K1735R). The western blot revealed with anti Flag antibodies shows that in p3X Flag SecPH 5K-R lane, bands 1 and 4 are enhanced, bands 3 is fainter and band 2 is lost. The nonspecific binding of unSUMOylated Flag SecPH derivatives to the cobalt beads is indicated by an asterisk.

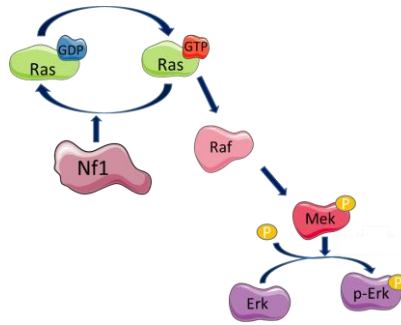
## Supplementary Figure 2



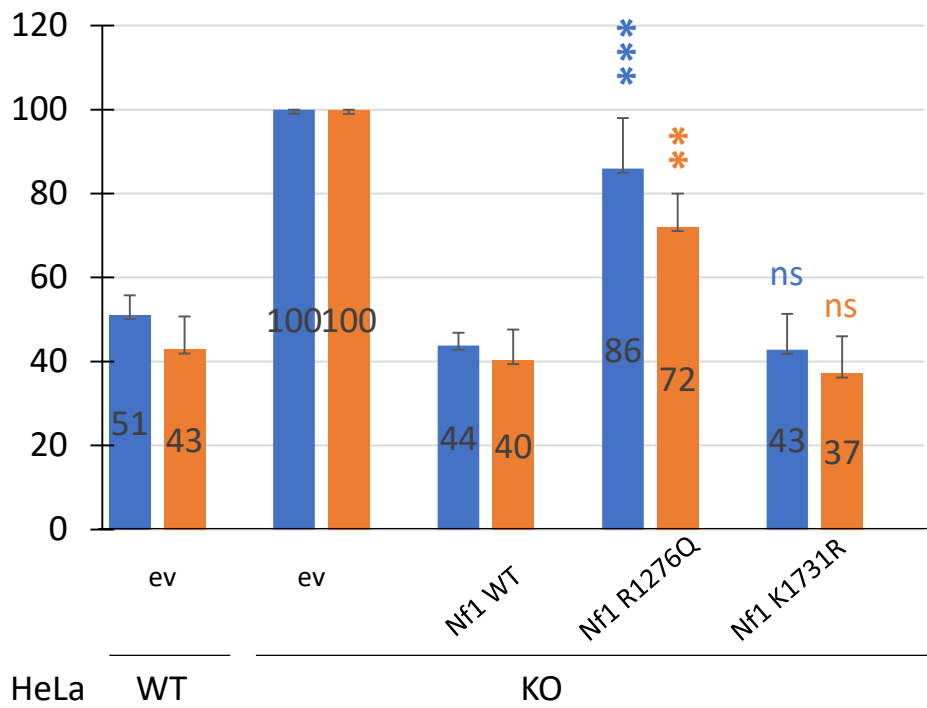
**Figure S2. Localisation of V1732 in SecPH 3D structure.** SecPH (PDB: 2E2X) is shown as cartoon (a) and surface (b) representations. Sec is in light blue and PH in pink. SCM residues (DTK<sub>1731</sub>V) are in blue (D1729 and T1730), red (K1731) and green (V1732). K1731 is shown as a stick in cartoon view.

Supplementary Figure 3

a



b



### **Figure S3. Ras-GAP activity of the Nf1 K1731R and Nf1 R1276Q mutants**

HeLa WT cells were transfected with p3X Flag (e.v.), and HeLa KO (NF1<sup>-/-</sup>) cells were transfected with p3X Flag (e.v.), p3X Flag Nf1, p3X Flag Nf1 K1731R or p3X Flag Nf1 R1276Q mutants (R1276Q is a pathogenic mutation in GRD c.3827G>A (p.Arg1276Gln) (Ref 65) known to abolish Ras-GAP activity). Note that in this experiment i) Ubc9 and SUMO2 were not overexpressed in order to prevent the SUMO-dependant Ras proteins activation and ii) 24 h after transfection, cells were grown for 18 h in serum-starved medium to decreased 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.

**a.** Schematic of the Ras/MEK/ERK pathway and of the Ras-GAP activity of Nf1 which activates the intrinsic GTPase activity of Ras. pERK/ERK ratio and Ras-GTP level will be determined to evaluate the Ras-GAP activity of Nf1.

**b.** Quantification pERK/ERK ratios (blue bars) and Ras-GTP level (orange bars) for the different cells analyzed in at least six independent experiments (n>6). pERK/ERK ratios were calculated after quantification of the immunoblots by densitometry using the Genetool software of Syngene. pERK/ERK ratio and Ras-GTP level of HeLa KO (NF1<sup>-/-</sup>) cells transfected with p3X Flag (e.v.) were set at 100% and all the other pERK/ERK ratios and Ras-GTP levels were reported relative to this value and indicated into the bars. SEM error bars are shown. Statistical analyses were performed using StatEL software ([www.adscience.eu](http://www.adscience.eu)). The non-parametric Mann-Whitney U test was used. The criterion of significance was set as 5% and the results were considered statistically significant at *p*-value <0.05 (\*), <0.01 (\*\*), or <0.001 (\*\*\*). ns stands for non significant.