Supplementary information

Supplementary Materials and Methods

Recombinant protein production and purification

6xHis-tagged NEMO derivatives and GST(Ub)4 proteins were produced in E.Coli Rosetta II strain (Novagen) using ZYM-5052 auto-induction culture medium (Studier, 2005) for 42 hours at 30ºC. 250 ml of bacterial culture were centrifuged and the pellets were resuspended in a buffer containing 50 mM Tris pH 7.5, 20 mM KCl, 0.2 mM DTE, 1 mg/ml and 5% glycerol. After sonication, Triton X-100 was added (1% final concentration) and following 1 hour rocking at 4ºC, lysates were centrifuged. The cleared bacterial lysates obtained were used for protein purification. NEMO polypeptides were purified using His-Trap columns (GE Healthcare) according to the manufacturer's instruction. Eluted proteins were dialyzed overnight at 4ºC against a buffer containing 50 mM Tris pH 8.0, 100 mM KCl, 0.2 mM DTE and 0.1 mM DDM. Glycerol was then added to 20% final concentration and proteins were stored at at -80ºC until use. For fluorescence measurements, recombinant proteins were further purified as previously described (Agou et al, 2002). GST(Ub)4 and GST(Ub)9 were produced similarly except that glutathione-agarose beads (Sigma) were used for purification. GST fusion proteins were either left bound to the beads and frozen at -20ºC in a buffer containing 50 mM Tris pH 7.5, 150 mM NaCl and 20% glycerol. Alternatively, the proteins were eluted from the beads with 20 mM reduced glutathione, 50mM Tris (pH 8,0), dialysed against a buffer containing 50mM Tris (pH 7,5), 150 mM NaCl and 0,5 mM DTE and frozen at -20ºC until use. Removal of the GST moiety was done by cleavage with sepharose-immobilized thrombin (Calbiochem) and GST was finally removed by further incubation with glutathione-agarose beads. Protein concentrations were determined by absorption at 280nm, and purity was verified by SDS-PAGE and Coomassie staining.

Fluorescence spectroscopy

The dissociation constants (K_D) of the CC2-LZ(F312W), NOA and NOAZ peptides were determined by fitting experimental curves using the Monte Carlo algorithm (pro Fit 6.1, Quantum Soft). Experimental data were fitted according to the following equations:

$$(F_{obs}-F_0)=(F_{max}-F_0)*(1-\alpha_{bound}) \quad (1)$$

where F_0 represents the initial fluorescence intensity of a fraction of CC2-LZ(F312W) bound to polyubiquitin chains (polyUb) in the absence of competitor, F_{max} is the fluorescence after saturation with the competitor (identical to the fluorescence of CC2-LZ(F312W) alone), and α_{bound} is the molar fraction of CC2-LZ(F312W) bound to polyubiquitin chains. In our competitive binding assays, as the free concentration of polyUb cannot be approximated to the total concentration, we used a general equation previously described in (Wang, 1995) to determine the free molar fraction of F312W ($1-\alpha_{bound}$), which can be expressed as:

$$1-\alpha_{bound} = [2\text{sqrt}(a^2-3b)\cos(\theta/3) - a]/[3K_D^{F312W} + (2\text{sqrt}(a^2-3b)\cos(\theta/3) - a] \quad (2)$$

where

$\theta = \arccos[(-2a^3 + 9ab - 27c)/(2\text{sqrt}((a^2-3b)^3))]$

$a = K_D^{F312W} + K_D + [F312W]_t + [competitor]_t - [PolyUb]_t$

$b = K_D([F312W]_t - [PolyUb]_t) + K_D^{F312W}([competitor]_t - [PolyUb]_t) + K_D^{F312W} K_D$

$c = - K_D^{F312W} K_D [PolyUb]_t$

$[PolyUb]_t$ and $[F312W]_t$ are total polyUb and CC2-LZ(F312W) concentration respectively, $[competitor]_t$ is the total concentration of CC2-LZ or NOAZ, K_D^{F312W} the dissociation constant of the CC2-LZ(F312W) and K_D the dissociation constant of the competitor (CC2-LZ or NOAZ).

The fitted parameters used in equations (1) and (2) were F_{max}, F_0, K_D^{F312W} and K_D. The K_D values which gave the best fitting for two independent concentrations of F312W (0.5 and 3 μM) are shown in Table 2.

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Supplementary figures

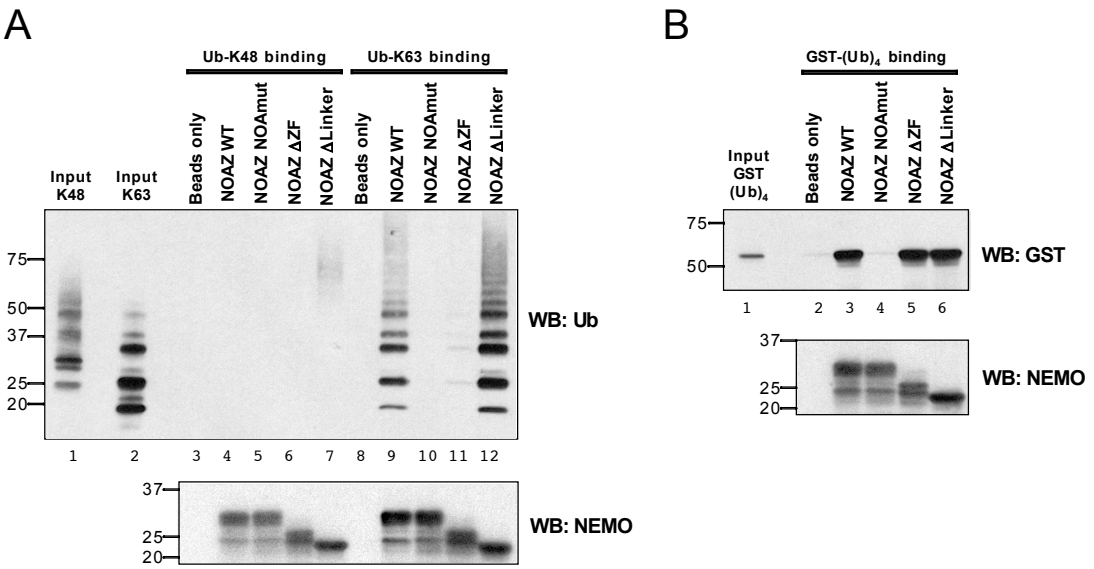

Figure S1 : **Mutations in the NOA domain or deletion of the Zinc Finger strongly reduce binding of NEMO to ubiquitin K63-linked chains, while binding to linear ubiquitin chains is only dependent on the NOA domain.** A) Histidine-tagged recombinant NOAZ domains of NEMO (purified as described in supplementary Materials and Methods) : WT (aa 215-412), NOAmut (215-412, Y301S D304N), ΔZF (215-386) and Δlinker (Δ339-386) bound to nickel-activated Sepharose beads were incubated with either K48- or K63-linked polyubiquitin chains (3-7 in length). After washing, recovered material was probed with an anti-ubiquitin antibody. B) Same as in A) except that binding reactions were done with $GST(Ub)_4$. Recovered material was probed with an anti-GST antibody. The amount of each recombinant protein used for the binding assays was verified by reblotting the membranes with an anti-NEMO antibody. The input lanes correspond to 8% of the material used for the binding reactions. Note: high molecular weight species pulled down with ubiquitin K63-linked chains (lane 9 and 12) are enriched long K63-linked chains already present in the commercial preparation (and visible in the input lane upon overexposure, data not shown).

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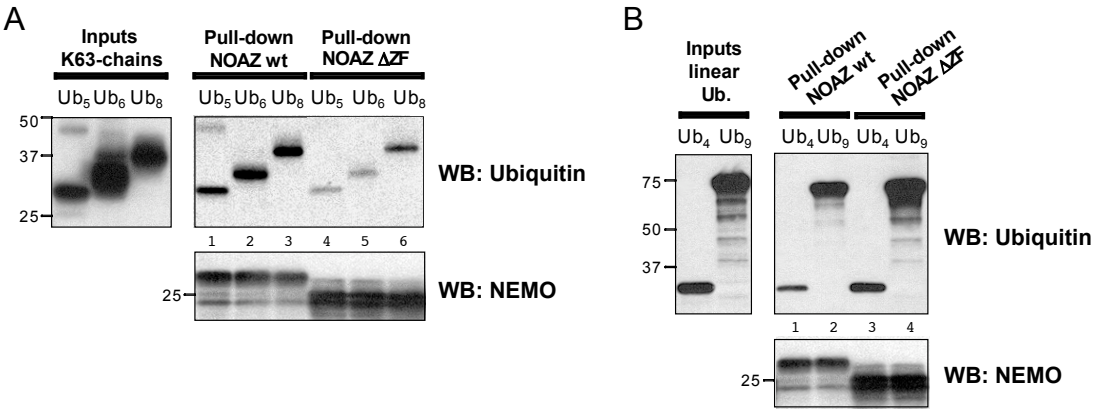

Figure S2 : **Pull-down experiments using recombinant NEMO polypeptides and fixed-length K63-linked or linear ubiquitin chains**. A) Histidine-tagged recombinant NOAZ domains (purified as described in supplementary Materials and Methods) : WT (aa 215-412) and ΔZF (215-386) bound to nickel-activated Sepharose beads were incubated with K63-linked penta-, hexa- and octa-ubiquitin (Ub_5, Ub_6 and Ub_8 respectively). After washing, recovered material was probed with an anti-ubiquitin antibody. B) Same as in A) except that binding reactions were done with linear tetra- and nona-ubiquitin (Ub_4 and Ub_9 respectively). The amount of each recombinant protein used for the binding assays was verified by reblotting the membranes with an anti-NEMO antibody. The input lanes correspond to 8% of the material used for the binding reactions. Note that binding to K63-linked ubiquitin chains requires the ZF domain of NEMO, while binding to linear ubiquitin chains is ZF-independent (irrespective of the chain length).

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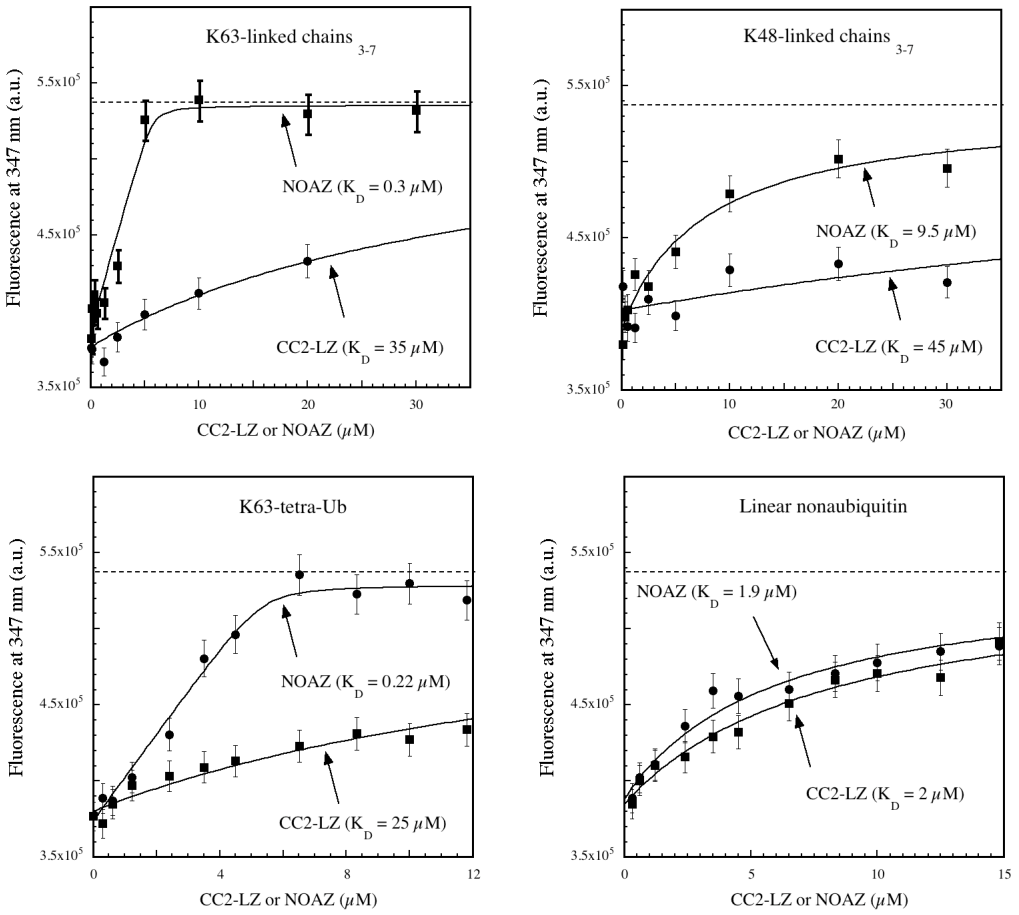

Figure S3 :**Measurement of the K_D values of the CC2-LZ and NOAZ domains for K48-, K63-polyubiquitin$_{3-7}$, K63 tetra-ubiquitin or linear nona-ubiquitin.** Binding competitions of polyubiquitin chains (3µM) to CC2-LZ (3µM) were performed by using variable concentrations of CC2-LZ or NOAZ as described in Materials and Methods. Solid line represents fitted curves allowing to calculate the K_D values presented in Table 2 (derived from EC_{50} as described in supplementary Materials and Methods), while dashed line indicate the fluorescence after saturation with the competitor (identical to the fluorescence of F312W alone).

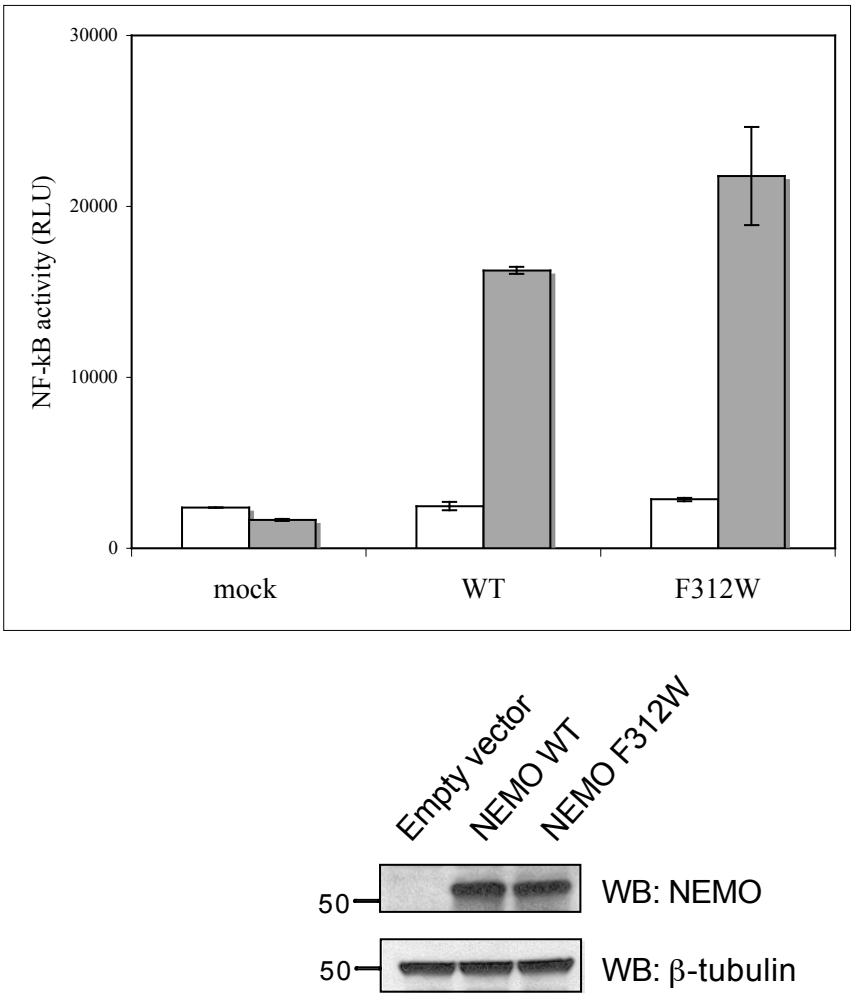

Figure S4 : **The F312W mutant of NEMO complements NF-κB-deficient cells as efficiently as wt NEMO**. NF-κB activation by TNF-α was measured in NEMO-deficient JM4.5.2 T cells following transfection with WT or F312W mutant NEMO together with a NF-κB-dependent firefly luciferase reporter construct and a control renilla luciferase-expressing plasmid. White bars : untreated cells, grey bars : TNF treated. A control western blot indicates that the level of expression of the WT and mutant NEMO proteins are identical.

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