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# **Lack of interaction between NEMO and SHARPIN impairs linear ubiquitination and NF- $\kappa$ B activation, and leads to incontinentia pigmenti**

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## Abstract

**Background:** Incontinentia pigmenti (IP; MIM308300) is a severe, male-lethal, X-linked, dominant genodermatosis resulting from loss-of-function mutations in the *IKBKG* gene encoding NF- $\kappa$ B essential modulator (NEMO, the regulatory subunit of the IKK complex). In 80% of cases of IP, the deletion of exons 4 to 10 leads to the absence of NEMO and total inhibition of NF- $\kappa$ B signaling. Here, we described a new *IKBKG* mutation responsible for IP resulting in an inactive truncated form of NEMO.

**Objectives:** To identify the mechanism(s) by which the truncated NEMO protein inhibits the NF- $\kappa$ B signalling pathway.

**Methods:** We sequenced the *IKBKG* gene in IP patients and performed complementation and transactivation assays in NEMO-deficient cells. We also used immunoprecipitation assays, immunoblotting and an *in situ* proximity ligation assay to characterize the truncated NEMO protein interactions with IKK- $\alpha$ , IKK- $\beta$ , TRAF6, TRAF2, RIP1, HOIL-1, HOIP and SHARPIN. Lastly, we assessed NEMO linear ubiquitination by immunoblotting and investigated the formation of NEMO-containing structures (using immunostaining and confocal microscopy) after cell stimulation with IL-1 $\beta$ .

**Results:** We identified a novel splice mutation in *IKBKG* (c.518+2T>G, resulting in an in-frame deletion: p.DelQ134\_R256). The mutant NEMO lacked part of the CC1 coiled-coil and the HLX2 helical domain. The p.DelQ134\_R256 mutation caused inhibition of NF- $\kappa$ B signaling, although the truncated NEMO protein interacted with proteins involved in the activation of NF- $\kappa$ B signaling. The IL-1 $\beta$ -induced formation of NEMO-containing structures was impaired in IP fibroblasts carrying the truncated NEMO form (as also observed in HOIL-1<sup>-/-</sup> cells). The truncated NEMO interaction with SHARPIN was impaired in a male fetus with IP, leading to defective linear ubiquitination. **Conclusion:** We identified a hitherto unreported disease mechanism (defective linear ubiquitination) in IP.

**Clinical implications:** A splice mutation in *IKBKG* gene responsible for IP result in an inactive

truncated form of NEMO, unable to interact specifically with SHARPIN leading to defect in NEMO linear ubiquitination.

**Key words:** *Incontinentia pigmenti, IKBKG, NEMO, NF- $\kappa$ B, LUBAC, linear ubiquitination, SHARPIN*

### **Abbreviations used**

IP: incontinentia pigmenti

NEMO: NF- $\kappa$ B essential modulator

NF- $\kappa$ B: nuclear factor  $\kappa$ B

IKK: I $\kappa$ B Kinase

ID: immunodeficiency

EDA-ID: anhidrotic ectodermal dysplasia with immunodeficiency

IL-1 $\beta$ : interleukin 1 beta

TNF- $\alpha$ : tumor necrosis factor alpha

LUBAC: linear ubiquitin assembly complex

SHARPIN: SHANK-associated RH domain-interacting protein

HOIP: HOIL-1 interacting protein

HOIL-1: hemo-oxidized IRP2 ligase 1

Ub: ubiquitin

OTULIN: OTU deubiquitinase with linear linkage specificity

### **Capsule Summary**

We identified for the first time in human the NEMO domain involved in SHARPIN interaction which is dispensable for NF- $\kappa$ B signaling. Beside, HOIL-1, HOIP, and OTULIN deficiency we confirmed that defect of linear ubiquitination is responsible for human diseases.

## Introduction

Incontinentia pigmenti (IP; MIM308300) is a severe, male-lethal, X-linked dominant genodermatosis. It is characterized by the occurrence of Blaschko-linear skin lesions during the neonatal period, in four successive stages: erythema, vesicles, pustules (stage 1), verrucous lesions (stage 2), hyperpigmentation (stage 3), and atrophy (stage 4)<sup>1</sup>. Neonatal rashes may be associated with developmental anomalies of the teeth, eyes, hair and central nervous system<sup>2-4</sup>. Early lethality in IP males and complete skewing of X-inactivation in IP females unequivocally result from null, loss-of-function mutations in the *IKBKG* gene coding for NF- $\kappa$ B essential modulator (NEMO/IKK- $\gamma$ , the regulatory subunit of the IKK complex, a core component of the NF- $\kappa$ B cascade). The downstream effector NF- $\kappa$ B is an ubiquitous transcription factor involved in many physiological functions, including inflammatory and immune responses, apoptosis, cell survival, and embryonic development<sup>5,6</sup>. 70 to 80% of IP patients carry a common genomic rearrangement that results in the deletion of exons 4 to 10 of the *IKBKG* gene<sup>7-9</sup>. Most of the other IP-associated *IKBKG* mutations identified to date are truncating mutations that result in complete inhibition of the NF- $\kappa$ B signaling pathway. Hypomorphic, non-lethal, missense mutations in *IKBKG* result in residual NF- $\kappa$ B activity and can cause isolated immunodeficiency (ID) or anhidrotic ectodermal dysplasia with ID syndrome (EDA-ID). The latter syndrome is combined with osteopetrosis and lymphedema in some cases and is isolated in other cases<sup>10-20</sup>.

In resting cells, NF- $\kappa$ B is trapped in the cytoplasm by inhibitory molecules from the I $\kappa$ B family. In response to a variety of stimuli (e.g. interleukin 1 $\beta$  (IL-1 $\beta$ ), bacterial lipopolysaccharide, and proinflammatory cytokines such as tumor necrosis factor alpha (TNF- $\alpha$ ), the IKK complex is activated and phosphorylates the inhibitory I $\kappa$ B proteins. The latter are then rapidly ubiquitinated and degraded by the 26S proteasome machinery. This subsequently enables the NF- $\kappa$ B transcription factor to translocate into the nucleus and regulate target gene transcription<sup>5,21,22</sup>.

It has recently been shown that upon stimulation by TNF- $\alpha$  or IL-1 $\beta$ , NEMO is rapidly and transiently recruited into punctuate structures<sup>23</sup>. The latter are enriched in activated IKK kinases and ubiquitinated

NEMO molecules. Furthermore to the K-63-linked polyubiquitination of NEMO<sup>24</sup>, it has been recently demonstrated that a novel type of head-to-tail-linked linear polyubiquitination is also critical for regulation of the NF- $\kappa$ B pathway<sup>25-29</sup>. Linear polyubiquitination is mediated *via* the linear ubiquitin chain assembly complex (LUBAC), a 600 kDa Ub ligase ternary complex composed of hemo-oxidized IRP2 ligase-1 (HOIL-1), HOIL-1-interacting protein (HOIP) and SH3 and multiple ankyrin repeat domains associated RH domain interacting protein (SHARPIN). In the present study, we generated functional data on fibroblasts derived from an IP male fetus carrying a c.518+2T>G splice mutation in the *IKBKG* gene. We found that loss of the NEMO-SHARPIN interaction impaired the recruitment of truncated NEMO forms into the punctuate structures that are transiently formed upon cell stimulation, and thus led to a defect in linear ubiquitination. Our results highlight a novel disease mechanism in IP.

## Results

### Genetic and functional analyses of the truncated NEMO protein

We assessed a large IP kindred segregating over three generations. All the affected females had a skewed X inactivation pattern (**Fig 1, A**) and presented with typical, IP-associated skin lesions. Female II.3 also suffered from right hemiplegia. Female II.8 had a history of seizures during infancy, persistent migraine attacks, and two early, spontaneous abortions (before 10 weeks of gestation). During the second of these pregnancies (III.4), an indirect molecular diagnosis (using DNA extracted from chorionic villus samples) identified the haplotype that segregated with IP disease in the family. A therapeutic abortion was performed. Fibroblasts from a skin biopsy of the affected male fetus were then cultured. We identified a splice donor site mutation in *IKBKG* exon 4 (c.518+2T>G, **Fig 1, B**), which segregated with the disease in the whole kindred. RT-PCR and then direct sequencing of RNAs from the affected male fetus's cultured skin fibroblasts confirmed the presence of the c.518+2T>G mutation, which resulted in the skipping of exons 4-6 (**Fig 1, C and D**). The c.518+2T>G mutation is predicted to produce a truncated protein that lacks part of the coiled-coil motif (CC1) and the NEMO linker (the HLX2 helical domain) but retains the C-terminal part of NEMO (**Fig 1, E**). Indeed,

Western blot analyses confirmed the presence of a truncated NEMO protein with a molecular weight of about 32 kDa.

The resulting truncated NEMO protein (p.DelQ134\_R256) thus contained all the essential domains. The deletion introduces a novel junction between Q133 (CC1) and G257 (CC2-LZ), which probably causes a discontinuity in the regular heptad repeats of the CC1 and CC2-LZ domains and may thus impair oligomerization. Furthermore, the shortened superhelical coiled-coil structure is predicted to change the orientation of the Ub-binding NEMO-optineurin-ABIN domain (NOA, also referred as UBAN and NUB) relative to the IKK- $\beta$  binding site, as indicated in **Fig 2** and **Supplementary Figures**.

As observed in fetal fibroblasts carrying the common *IKBKG* deletion (Del exons 4-10, Fetus NEMO-<sup>-/-</sup>), fibroblast cells carrying the c.518+2T>G *IKBKG* mutation displayed severely impaired I $\kappa$ B $\alpha$  degradation following TNF- $\alpha$  and IL-1 $\beta$  stimulation (**Fig. 3, A**). Analysis (using ELISAs) of the secreted NF- $\kappa$ B target proteins showed that fibroblasts carrying the c.518+2T>G *IKBKG* mutation produced neither interleukin-6 (IL-6) nor interleukin-8 (IL-8) upon stimulation by TNF- $\alpha$  and IL-1 $\beta$  (**Fig 3, B**). In order to determine whether impaired NF- $\kappa$ B signaling was due to protein truncation alone or to putative NEMO isoforms resulting from the splice mutation, we subcloned the wild-type and truncated NEMO constructs and performed transactivation experiments in *Ikbkg*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs). The truncated p.DelQ134\_R256 NEMO isoform was unable to activate NF- $\kappa$ B in response to either TNF- $\alpha$  or IL-1 $\beta$  (**Fig 3, C**) - demonstrating that this event was sufficient for the complete inhibition of NF- $\kappa$ B activity. Hence, the c.518+2T>G genotype is a loss-of-function *IKBKG* mutation that inactivates the NEMO protein and impairs the NF- $\kappa$ B signaling pathway.

### **Integration of the truncated NEMO protein into the IKK complex, K63-polyubiquitination and oligomerization**

After the transfection of HEK293T cells with various combinations of constructs, immunoprecipitation (using anti-IKK- $\alpha$  or anti-IKK- $\beta$  antibodies) and Western blot analyses (using an anti-hemagglutinin (HA) antibody) showed that HA-tagged truncated NEMO protein was correctly

integrated into the IKK complex (**Fig 3, D**). Furthermore, immunoprecipitation (using specific antibodies against RIP1, TRAF2 and TRAF6) and immunoblots (using an anti-HA antibody) showed that the truncated NEMO species was also able to interact with RIP1, TRAF2 and TRAF6 – all of which are known to be required for NF- $\kappa$ B activation by TNF- $\alpha$  (RIP1 and TRAF2) or IL-1 $\beta$  (TRAF6) (**Fig 3, E**). In the latter experiment, the same results were obtained when immunoprecipitation was performed with an anti-HA antibody and immunoblotting was performed with the specific antibodies (**Fig 3, E**). It is noteworthy that TRAF6 is an E3 Ub ligase that is required for NF- $\kappa$ B activation and that catalyzes the K63-linked polyubiquitination of NEMO.

Co-transfection of HEK293T cells with NEMO plasmids and plasmids expressing ubiquitin in the presence of TRAF6 resulted in a similar K63-linked polyubiquitination pattern for both the truncated and wild-type NEMO proteins (**Fig 3, F**). In contrast, homodimerization/oligomerization of the truncated NEMO proteins was less intense (relative to control experiments) when FLAG-NEMO and HA-NEMO were co-transfected into HEK293T cells, immunoprecipitated using an anti-FLAG antibody and immunoblotted with an anti-HA antibody (**Fig 3, G**).

#### **The NEMO/LUBAC interaction**

We next assessed the interaction between HA-tagged wild-type NEMO or truncated NEMO proteins and each of the three LUBAC subunits (HOIL-1, HOIP and SHARPIN). Immunoprecipitation (using an anti-HA antibody) and immunoblotting (using anti-FLAG or anti-V5 tag antibodies) showed that wild-type NEMO interacted with all three human LUBAC subunits. In contrast, the truncated NEMO interacted with HOIL-1 and HOIP but not with SHARPIN (**Fig 4, A**).

In order to further characterize the interaction between NEMO and SHARPIN in cells, we used the Duolink II *in situ* proximity ligation assay (PLA); this is a highly specific, sensitive technique for detecting protein colocalization/interactions through the generation of localized spot signals<sup>30</sup>. An *in situ* PLA performed on transfected cells also provides the exact position of the recognition event. The maximum distance between two signal-producing epitopes ranges from 10 to 30 nm. Our PLA results showed that wild-type NEMO interacts directly with SHARPIN and HOIL-1 but not with HOIP (in

contrast to the above-mentioned immunoblot results (**Fig 4, A**); see the Discussion). The truncated NEMO form was also able to interact with HOIL-1 but failed to bind to SHARPIN (**Fig 4, B**) - thus confirming the immunoblot results in this respect.

#### **Formation of supramolecular NEMO-containing complexes and linear ubiquitination in the patients' fibroblasts in response to IL-1 $\beta$**

We recently showed that the activated IKK complex is rapidly and transiently reorganized into supramolecular complexes in response to stimulation by cytokines such as IL-1 $\beta$  (marking the earliest steps of NF- $\kappa$ B activation)<sup>23</sup>. We therefore used immunostaining and confocal microscopy to investigate the formation of NEMO-containing structures in IP cells upon IL-1 $\beta$  stimulation. We found that the IL-1 $\beta$ -induced formation of NEMO-containing structures was impaired in (i) IP fetus III.4 fibroblasts carrying the truncated NEMO form (Del 134\_326), (ii) IP fetus fibroblasts carrying the frequent *IKBKG* gene rearrangement (Del exons 4-10 ; NEMO-/-) and (iii) recently characterized HOIL-1-deficient fibroblasts from non-IP patients (**Fig 5, A**). As expected, the stimulation of control cells with IL-1 $\beta$  yielded dots of NEMO- and SHARPIN-containing supramolecular structures. In contrast, the IL-1 $\beta$ -induced recruitment of these supramolecular complexes was impaired in cultured fibroblasts obtained from various IP patients (**Fig 5, A**). Furthermore, quantification of the immunostained dots revealed low levels of SHARPIN in fibroblasts carrying the truncated form of NEMO (relative to fibroblasts from healthy controls ; **Fig 5, B**). The same results were found in HOIL-1-deficient fibroblasts and in NEMO-deficient IP cells carrying the *IKBKG* gene rearrangement. Moreover, immunoblot assays revealed low expression levels of HOIL-1, HOIP and SHARPIN in IP fetus III.4 cells (relative to control cells ; **Fig 5, C**). RT-QPCR analyses showed that mRNA levels expression of the three LUBAC subunits are similar in IP fetus III.4 and control fibroblasts (**Fig 5, D**).

Lastly, we used immunofluorescence assays with a recently engineered anti-linear Ub chain antibody to examine the formation of linear polyubiquitin chains in IL-1 $\beta$ -treated fibroblasts carrying the truncated NEMO form<sup>31</sup>. Interestingly, we found that linear polyubiquitin aggregates colocalized with NEMO in IL-1 $\beta$ -treated control cells (**Fig 6, A**). These aggregates were weakly present in IP

cells and in HOIL-1-deficient fibroblasts from non-IP patients. Quantification of NEMO and linear Ub chains confirmed these observations (**Fig 6, B**). Co-transfection of HEK293T cells with NEMO plasmids and plasmids encoding ubiquitin KO (where all Lysine residues were replaced by Arginine) and LUBAC subunits, showed a defect of linear ubiquitination of the truncated NEMO protein (Fig 6, C). These findings suggest that perturbation of the NEMO-SHARPIN interaction impairs linear ubiquitination of NEMO.

## Discussion

Here, we showed that a novel *IKBKG* splice site mutation in an affected male fetus from a large IP kindred resulted in truncation of the NEMO protein CC1 and HLX2 domains. The truncated form of NEMO dimerizes/oligomerizes less readily than the wild type. Moreover, the interaction between the truncated NEMO protein and SHARPIN is specifically impaired and results in defective linear ubiquitination. In contrast, the truncated NEMO protein interacted normally with HOIL-1, HOIP, RIP1, TRAF2 and TRAF6. K63-linked polyubiquitination of truncated NEMO by TRAF6 was normal, and the mutant protein was still recruited into the IKK complex.

Transfection/immunoprecipitation assays and PLAs provided evidence of loss of interaction of the truncated form of NEMO with SHARPIN and maintenance of the interaction with HOIP and HOIL-1. Given that the PLAs did not provide any evidence of a close interaction between wild-type NEMO and HOIP (in contrast to the immunoblots), we suggest that these two proteins interact through SHARPIN, HOIL-1 or Ub chains in humans. This would differ from the situation in mice, where HOIP binds directly to NEMO via the HOIP NZF1 domain. Moreover, we found that the IL-1 $\beta$ -induced formation of NEMO-containing structures in fibroblasts carrying the truncated NEMO protein was impaired (as has already been reported for HOIL-1 and HOIP-deficient fibroblasts)<sup>32,33</sup>. Furthermore, we showed that the colocalization of linear polyubiquitin aggregates and NEMO is severely impaired in fibroblasts carrying the truncated NEMO protein (as in HOIL-1 and NEMO-deficient fibroblasts) treated with IL-1 $\beta$ . Immunoblot analyses confirmed the defect of linear ubiquitination of the truncated NEMO protein. Lastly, we observed abnormally low expression of all

three LUBAC subunits in IP fibroblasts carrying the truncated NEMO species. Based on these results, we suggest that NEMO is essential for the stabilization of LUBAC at the protein level.

Otherwise, concerning the other major disease associated to *IKBKG* mutations, the anhidrotic ectodermal dysplasia with immunodeficiency, we cannot rule out the possibility that the responsible hypomorphic mutations located in the Q134-R256 deleted domain could impair the interaction between NEMO and SHARPIN. Indeed, the *panr2* mouse described below carrying the L153P hypomorphic mutation in *Ikbkg* gene impairs the interaction between Nemo and Sharpin<sup>34</sup>. Interestingly, an EDA-ID patient has been reported to carry a mutation in the human *IKBKG* gene that affects the same amino acid that is mutated in the *panr2* mouse (L153R)<sup>18</sup>. Hence, one cannot rule out the possibility that the human L153R mutation impairs the NEMO-SHARPIN interaction as it does in mice. Thus, a defect in interaction between NEMO and SHARPIN is an attractive mechanism for both IP and EDA.

Non-degradative, linear ubiquitination has recently emerged as a key mediator of NF- $\kappa$ B signaling. This LUBAC-mediated process is involved in the NF- $\kappa$ B pathway responses to both TNF- $\alpha$  and IL-1 $\beta$ . Linear polyubiquitin chains are conjugated to specific Lys residues in NEMO and RIP1. This activity is essential for full NF- $\kappa$ B activation and has a key role in inflammatory and immune responses in both mice and humans<sup>25-29</sup>. Based on findings in both human and mice, the three subunits of LUBAC could have functions independently of LUBAC enzymatic activity. For instance, *Hoip*<sup>-/-</sup> mice are lethal whereas *Hoil-1*<sup>-/-</sup> mice are viables and present immunodeficiency<sup>35,36</sup>. *Sharpin*-deficient mice is characterized by a chronic proliferative dermatitis and a multiorgan inflammation, phenotype further more different from the two others<sup>37, 38</sup>. Moreover, in our immunofluorescence experiments, in addition to their cytoplasmic localization, both NEMO and SHARPIN have been found to interact into the nucleus (**Fig 4, B**), which is consistent with SHARPIN function(s) independently to LUBAC complex.

In human, the link between NF- $\kappa$ B and autoinflammation was suggested by the recent demonstration that genetic deficiency of HOIL-1, HOIP and OTULIN proteins (involved in regulation of NEMO linear ubiquitination) arises in atypical autoinflammatory diseases<sup>32,33,39</sup>. In mice, deficiency of *Sharpin*

also results in an autoinflammatory phenotype<sup>37,38</sup>. However, a direct link between NEMO and autoinflammation in human has not been previously demonstrated. We suggest that autoinflammation as well as immunodeficiency and may be other unidentified symptoms are probably masked via the total skewed X-inactivation, a hallmark of IP females. Since NF- $\kappa$ B protect against apoptosis, the total skewed X-inactivation is actually the results of complete elimination, shortly after birth, of cells with the active X chromosome bearing the mutated *IKBKG* allele.

To the best of our knowledge, the link between NF- $\kappa$ B and autoinflammation has been highlighted by publications of IP females displaying Behcet syndrome<sup>40,41</sup> an authentic multisystem autoinflammatory disease. Moreover, a heterozygous D406V mutation specifically in the *IKBKG* gene was identified in an IP female with Behcet's syndrome<sup>42</sup>. In the latter case, the X-inactivation was not completely skewed, which suggests that cells carrying the active mutated X-chromosome had not been completely eliminated, probably because the *IKBKG* mutation is less severe. Furthermore, an in-frame, *de novo* deletion of 51 amino acids in NEMO protein was found to result in a deficient IRF3-mediated response, constituting a novel mechanism for autoinflammatory disease via NEMO (ACR/ARHP Annual Meeting, 2013).

Another *IKBKG* splice mutation (c.518+5G>A) has been reported in a severe form of EDA-ID<sup>43</sup>. It was also predicted to result in the skipping of exons 4-6 and thus a similarly truncated form of NEMO protein. In fact, this genotype corresponded to a less harmful, non-lethal EDA-ID phenotype in which some normal splicing of the *IKBKG* mRNA probably persisted. These normal *IKBKG* mRNA molecules probably provided some residual NF- $\kappa$ B activity and rendered the mutation non-lethal.

SHARPIN is a key regulator of immune and inflammatory responses and cytokine production. It is also involved in TNF- $\alpha$ -dependent NF- $\kappa$ B activation and anti-apoptotic signaling in hepatocytes<sup>44</sup>. Interestingly, the spontaneous *Sharpin*-deficient mutation in mice (the *cpdm* mouse) resulted in epidermal hyperplasia and inflammation with the presence of apoptotic bodies in keratinocytes and elevated rates of cell death in the skin (as also observed in *Ikbkg*<sup>-/-</sup> mice and in IP)<sup>37,38</sup>. Although

*SHARPIN* mutations have never been described in humans, they may conceivably be associated with a complex inflammatory phenotype with skin involvement.

In conclusion, we demonstrated that the interaction between a truncated NEMO protein and *SHARPIN* (but not HOIL-1 or HOIP) was specifically impaired in fibroblasts obtained from a male fetus with IP. Defective linear ubiquitination of NEMO may therefore constitute a hitherto unreported disease mechanism in IP.

## Material and Methods

**Case reports.** Carrier females and most of their unaffected relatives underwent a full clinical examination. All females with IP met the 1993 revised criteria for the classification of IP. The females with IP had a history of perinatal blistering and at least one of the other skin lesion stages. Several early, spontaneous abortions were reported, although most had not been investigated further. Biological material was only collected after the patients' had given their written, informed consent, in accordance with the tenets of the Declaration of Helsinki.

**Linkage analysis.** DNA was extracted from peripheral blood lymphocytes or (for the IP fetus III.4) fibroblasts via conventional phenol-chloroform purification. Linkage analysis was performed using fluorescent polymorphic microsatellite markers (p39, DXS1073 and F8c). After amplification, PCR products were mixed with the GeneScan 400D ROX size standard ladder (PE Applied Biosystems, Foster City, CA) and analyzed with an automatic sequencer (ABI PRISM 3130™, Applied Biosystems) using Genotyper™ and GeneScan™ software packages (Applied Biosystems).

**X-inactivation analysis.** X-inactivation patterns in the patients' peripheral blood lymphocytes were determined by PCR amplification of the androgen receptor locus. The procedures for digestions and PCR have been described elsewhere<sup>45</sup>. PCR products were migrated on an automatic sequencer (ABI PRISM 3130™) with GeneScan 400D ROX size standard ladder (PE Applied Biosystems, Foster City, CA), and analyzed using the Genotyper™ and GeneScan™ software packages.

**Sequencing.** All of the *IKBKG* gene exons and flanking intronic sequences were amplified by PCR with specific primers. Both DNA strands were sequenced using the Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems). PCR products were migrated on an ABI PRISM 3130™ sequencer (Applied Biosystems) and analyzed with Seqscape Analysis software. Sequence variations of *IKBKG* were numbered with the adenine of the ATG initiation codon as the first nucleotide (GenBank accession NM\_003639.3).

**RT-PCR and real time PCR.** Total RNA was isolated from cultured fibroblasts (derived from a male fetus with IP or from healthy controls) using an RNeasy® Plus Minikit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. RNA samples were reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems). Reverse transcript (RT)-real time PCR was carried out using the Power SYBR Green PCR Master Mix (PE Applied) on an ABI prism 7000 (PE Applied Biosystems), in triplicate. Dissociation analysis for each primer pair and reaction was performed to verify specific amplification.

**Structural modeling of the fully dimeric NEMO and its mutant (Del 134-256) complexed with IKK-β and linear or K63-di-Ubs.** The complex comprising human dimeric NEMO protein (residues 49-419) and linear or K63-di-Ub chains was modeling using the COILS<sup>46</sup> and PAIRCOIL2<sup>47</sup> coiled-coil prediction algorithms and the following crystal and NMR structures of NEMO fragments: KBD (PDB ID: 3BRV, residues 49-109); HLX2 (PDB ID: 3CL3, residues 193-249), CC2-LZ (PDB ID: 2V4H and 4BWN, residues 257-344) and ZF (PDB ID: 2JVX, residues 392-419). All the regular heptad phases (abcdefg)<sub>n</sub> within the predicted coiled-coil fragment of NEMO (residues 49-364) and common coiled-coil discontinuities such as stutters (deletion of three residues) and stammers (deletion of four residues) were correctly predicted by PAIRCOIL2, when compared with the heptad phases identified from the available crystal structures by the coiled-coil analysis program SOCKET<sup>48</sup>. This included (for example) a stutter at position 103 and a stammer at position 295, respectively. Two other highly predictable discontinuities at position 203 (a stutter) and 256 (the deletion of two residues) were taken into account to accommodate the heptad phases at the KD/HLX2 and HLX2/CC2-LZ junctions. The first model was built by assembling crystal and NMR NEMO fragments with the

following modeled regions: 110-192 (CC1, 83 aa), 250-256 (7 aa), 345-364 (20 aa) and 365-391 (27 aa). The long WT CC1 fragment (110-192) and the short mutant CC1 fragment (110-133) were generated with CC Builder, using a radius of 5.07 Å, a pitch of 225.8 Å and an interface angle of 26.42°<sup>49</sup>. The model of the linker region between CC2-LZ and ZF domains (365-391) was based on the CD spectra analyzes of the 248-344, 248-419 and 392-419 constructs of human NEMO (unpublished data). This 365-391 fragment was generated manually and refined from initial atomic coordinates derived from a I-TASSER-generated model based on a threading algorithm<sup>50</sup>. The NEMO:IKK-β: K63-diUb: linear-di-Ub complexes (wild type and mutant) were built by homology modeling; the structural templates were the crystal structure of the mouse CC2-LZ complexed with a linear di-Ub chain (PDB ID: 2ZVO) and the modeled NEMO ZF complexed with a K63 di-Ub chain. The short C-terminal fragment of IKK-β (i.e. the NEMO binding domain) bound to the N-terminus of NEMO had already been described as residues S705-Q743 in PDB ID: 3BRV<sup>51</sup>. PyMOL (version 1.7, Schrödinger) was used for molecular display and structural figures. Ubiquitin K0 cDNA (were all Lysine residues were replaced by Arginine, generous gift from R. Baer, Columbia University, New York) was subcloned into pcDNA3.1.

**Plasmid constructions.** Human cDNA containing the entire open reading frame or truncated cDNA of *NEMO* was amplified respectively from control RNA or fibroblast RNA from a male fetus with IP. The amplified DNA was then cloned into the pcDNA 3.1 expression vector, with either an HA epitope or a FLAG epitope N-terminal tag.

**Reagents.** The following antibodies were used: anti-NEMO (BD Biosciences Pharmingen, San Jose, CA, USA), anti-IκBα (sc-371, Santa Cruz Biotechnology, Inc.), anti-IKK-α (BD Biosciences Pharmingen), anti-IKK-β (BD Biosciences Pharmingen), anti-RIP (BD Biosciences Pharmingen), anti-TRAF6 (sc-7221, Santa Cruz Biotechnology, Inc.), anti-TRAF2 (sc-876, Santa-Cruz Biotechnology, Inc.), anti-SHARPIN (14626-1-AP; ProteinTech, IL, USA), anti V5 (Sigma Aldrich, MO, USA), anti-FLAG (Sigma Aldrich), anti HA (Sigma Aldrich). The specific human antibody against linear polyubiquitin chains (1F11/3F5/Y102L) was provided by Genentech (CA, USA).

**Fibroblast stimulation and IL-8/IL-6 production.** Control and patient-derived fibroblasts were immortalized by transfection with a plasmid encoding the SV40 large-T antigen. Transformed cell lines were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Invitrogen). Immortalized cells were grown in 24-well plates and incubated with 10 ng/ml of IL-1 $\beta$  or TNF- $\alpha$  (R&D Systems, MN, USA). 24 h after stimulation, supernatants were collected and IL-8 or IL-6 production was assessed with an ELISA, according to the manufacturer's instructions (Sanquin, Amsterdam, The Netherlands).

**Cell line complementation and transactivation assays.** Nemo<sup>-/-</sup> MEFs in 300  $\mu$ l of PBS were transiently transfected by electroporation (500  $\mu$ M and 160V, with a Gene Pulser X-cell Electroporation System, BioRad, Hercules, CA, USA) with 200 ng of either full length or truncated NEMO expression plasmid and 200 ng of pIgk-luc plasmid carrying the firefly luciferase gene under the control of a NF- $\kappa$ B promoter. pRenilla vector (100 ng) carrying the *Renilla* luciferase gene under the control of a ubiquitous promoter was used to normalize the transfection efficiency. After electroporation, cells were resuspended in DMEM/10% FCS and plated in 6-well dishes. Cells were serum-starved 24 hours later by adding DMEM without FCS. After 16 h of starvation, cells were stimulated with buffer alone, TNF- $\alpha$  (10 ng/ml) or IL-1 $\beta$  (10 ng/ml). Four hours later, cells were recovered in 100  $\mu$ l of lysis buffer (25 mM Tris-phosphate pH 7.8, 8 mM MgCl<sub>2</sub>, 1 mM DTT, Triton X-100, 25% glycerol) and firefly and *Renilla* luciferase activities were measured according to the manufacturer's instructions for the dual-luciferase system (Promega, Madison, WI, USA).

**Immunoprecipitation.** 2x10<sup>6</sup> HEK293T cells were transiently transfected (using jetPRIME<sup>TM</sup> reagent; Polyplus-transfection Inc., New York, NY, USA) with mutant or wild-type *NEMO* constructs (2  $\mu$ g per dish) and various partners of interest (2  $\mu$ g). 24 h after transfection, cells were lysed in special buffer (50 mM Tris-HCl pH 8, 170 mM NaCl, 0.5% NP-40, 50 mM NaF) with protease inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Immunoprecipitation was performed using  $\mu$ MACS HA Isolation Kit or  $\mu$ MACS protein G Microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Bound antibodies were visualized with horseradish-peroxidase-conjugated antibodies against rabbit or mouse IgG (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and the

Enhanced Chemiluminescence Kit with SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA)

***In situ* PLAs of LUBAC compounds with wild-type and NEMO truncated forms in cultured cells.** *In situ* PLAs (Duolink *in situ* PLA, from Olink Biosciences, Uppsala, Sweden) were carried out according to the manufacturer's instructions. Images were acquired and processed with Zeiss LSM-700 and Zen software.

**Immunofluorescence.** Cells cultured on glass coverslips were treated twice with ice-cold saponin extraction buffer (80 mM PIPES, pH 6.8, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, and 0.1% saponin) for 2 min and 4 min on ice, as previously described<sup>23</sup>. The cells were then washed twice with PBS before fixation in 100% methanol. The cells were progressively rehydrated by successive washes with 85%, 70%, and 50% ethanol in water and two final washes with PBS. For linear polyubiquitin staining, slides were treated with vOTU deubiquitinase (1 hour at 37°C). This eliminates unspecific interactions with the anti-linear-Ub chain antibody, since vOTU cleaves all Ub chain linkages other than linear chains. After blocking by incubation with 1% BSA in PBS, the slides were incubated with rabbit anti-SHARPIN, mouse anti-NEMO or human anti-linear polyubiquitin antibody, washed with PBS and incubated either with anti-rabbit Alexa Fluor 488 or anti-human Alexa Fluor 546 secondary antibodies (Life Technologies). The slides were washed again and incubated with DAPI (Sigma-Aldrich) to stain the nuclei. The coverslips were mounted in Mowiol supplemented with an anti-fading agent (AF100; Biovalley). Image acquisition was performed with Zeiss LSM 700 (Carl Zeiss Microscopy GmbH, Oberkochen, Germany) and Zen Software (Carl Zeiss Microscopy GmbH). For the quantification of NEMO, SHARPIN and linear Ub foci (presented in Fig 5 and 6), randomly selected fields acquired with a 63× objective lens and Z-stack were processed with ICY open source software and the Spot Detector tool ([icy.bioimageanalysis.org/](http://icy.bioimageanalysis.org/)).

## Bibliography

1. Landy SJ, Donnai D. Incontinentia pigmenti (Bloch-Sulzberger syndrome). *J Med Genet* 1993;30:53–9.

2. Hadj-Rabia S, Rimella A, Smahi A, Fraïtag S, Hamel-Teillac D, Bonnefont J-P, et al. Clinical and histologic features of incontinentia pigmenti in adults with nuclear factor- $\kappa$ B essential modulator gene mutations. *J Am Acad Dermatol* 2011;64:508–15.
3. Fusco F, Fimiani G, Tadini G, Michele D, Ursini MV. Clinical diagnosis of incontinentia pigmenti in a cohort of male patients. *J Am Acad Dermatol* 2007;56:264–7.
4. Minić S, Trpinac D, Obradović M. Systematic review of central nervous system anomalies in incontinentia pigmenti. *Orphanet J Rare Dis* 2013;8:25.
5. Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* 2008;8:837–48.
6. Li Q, Verma IM. NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2002;2:725–34.
7. Smahi A, Courtois G, Vabres P, Yamaoka S, Heuertz S, Munnich A, et al. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. *Nature* 2000;405:466–72.
8. Fusco F, Paciolla M, Napolitano F, Pescatore A, D'Addario I, Bal E, et al. Genomic architecture at the Incontinentia Pigmenti locus favours de novo pathological alleles through different mechanisms. *Hum Mol Genet* 2012;21:1260–71.
9. Aradhya S, Woffendin H, Jakins T, Bardaro T, Esposito T, Smahi A, et al. A recurrent deletion in the ubiquitously expressed NEMO (IKK-gamma) gene accounts for the vast majority of incontinentia pigmenti mutations. *Hum Mol Genet* 2001;10:2171–9.
10. Zonana J, Elder ME, Schneider LC, Orlow SJ, Moss C, Golabi M, et al. A novel X-linked disorder of immune deficiency and hypohidrotic ectodermal dysplasia is allelic to incontinentia pigmenti and due to mutations in IKK-gamma (NEMO). *Am J Hum Genet* 2000;67:1555–62.
11. Döffinger R, Smahi A, Bessia C, Geissmann F, Feinberg J, Durandy A, et al. X-linked anhidrotic ectodermal dysplasia with immunodeficiency is caused by impaired NF-kappaB signaling. *Nat Genet* 2001;27:277–85.
12. Jain A, Ma CA, Liu S, Brown M, Cohen J, Strober W. Specific missense mutations in NEMO result in hyper-IgM syndrome with hypohidrotic ectodermal dysplasia. *Nat Immunol* 2001;2:223–8.
13. Dupuis-Girod S, Corradini N, Hadj-Rabia S, Fournet J-C, Faivre L, Le Deist F, et al. Osteopetrosis, lymphedema, anhidrotic ectodermal dysplasia, and immunodeficiency in a boy and incontinentia pigmenti in his mother. *Pediatrics* 2002;109:e97.
14. Ku C-L, Dupuis-Girod S, Dittrich A-M, Bustamante J, Santos OF, Schulze I, et al. NEMO mutations in 2 unrelated boys with severe infections and conical teeth. *Pediatrics* 2005;115:e615–9.
15. Ku C-L, Picard C, Erdös M, Jeurissen A, Bustamante J, Puel A, et al. IRAK4 and NEMO mutations in otherwise healthy children with recurrent invasive pneumococcal disease. *J Med Genet* 2007;44:16–23.
16. Mooster JL, Cancrini C, Simonetti A, Rossi P, Di Matteo G, Romiti ML, et al. Immune deficiency caused by impaired expression of nuclear factor-kappaB essential modifier (NEMO) because of a mutation in the 5' untranslated region of the NEMO gene. *J Allergy Clin Immunol* 2010;126:127–32.e7.
17. Orange JS, Levy O, Brodeur SR, Krzewski K, Roy RM, Niemela JE, et al. Human nuclear factor kappa B essential modulator mutation can result in immunodeficiency without ectodermal dysplasia. *J Allergy Clin Immunol* 2004;114:650–6.

18. Hanson EP, Monaco-Shawver L, Solt LA, Madge LA, Banerjee PP, May MJ, et al. Hypomorphic nuclear factor-kappaB essential modulator mutation database and reconstitution system identifies phenotypic and immunologic diversity. *J Allergy Clin Immunol* 2008;122:1169–77.e16.
19. Puel A, Picard C, Ku C-L, Smahi A, Casanova J-L. Inherited disorders of NF-kappaB-mediated immunity in man. *Curr Opin Immunol* 2004;16:34–41.
20. Picard C, Casanova JL, Puel A. Infectious diseases in patients with IRAK-4, MyD88, NEMO, or IkB $\alpha$  deficiency. *Clin Microbiol Rev.* 2011 Jul;24(3):490-7. Review.
21. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* 2000;18:621–63.
22. Scheidereit C. IkappaB kinase complexes: gateways to NF-kappaB activation and transcription. *Oncogene* 2006;25:6685–705.
23. Tarantino N, Tinevez J-Y, Crowell EF, Boisson B, Henriques R, Mhlanga M, et al. TNF and IL-1 exhibit distinct ubiquitin requirements for inducing NEMO-IKK supramolecular structures. *J Cell Biol* 2014;204:231–45.
24. Deng L, Wang C, Spencer E, Yang L, Braun A, You J, et al. Activation of the IkappaB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell* 2000;103:351–61.
25. Tokunaga F, Sakata S, Saeki Y, Satomi Y, Kirisako T, Kamei K, et al. Involvement of linear polyubiquitylation of NEMO in NF-kappaB activation. *Nat Cell Biol* 2009;11:123–32.
26. Gerlach B, Cordier SM, Schmukle AC, Emmerich CH, Rieser E, Haas TL, et al. Linear ubiquitination prevents inflammation and regulates immune signalling. *Nature* 2011;471:591–6.
27. Ikeda F, Deribe YL, Skånland SS, Stieglitz B, Grabbe C, Franz-Wachtel M, et al. SHARPIN forms a linear ubiquitin ligase complex regulating NF- $\kappa$ B activity and apoptosis. *Nature* 2011;471:637–41.
28. Tokunaga F, Iwai K. Linear ubiquitination: a novel NF- $\kappa$ B regulatory mechanism for inflammatory and immune responses by the LUBAC ubiquitin ligase complex. *Endocr J* 2012;59:641–52.
29. Tokunaga F, Iwai K. LUBAC, a novel ubiquitin ligase for linear ubiquitination, is crucial for inflammation and immune responses. *Microbes Infect Inst Pasteur* 2012;14:563–72.
30. Söderberg O, Leuchowius K-J, Gullberg M, Jarvius M, Weibrecht I, Larsson L-G, et al. Characterizing proteins and their interactions in cells and tissues using the in situ proximity ligation assay. *Methods San Diego Calif* 2008;45:227–32.
31. Matsumoto ML, Dong KC, Yu C, Phu L, Gao X, Hannoush RN, et al. Engineering and structural characterization of a linear polyubiquitin-specific antibody. *J Mol Biol* 2012;418:134–44.
32. Boisson B, Laplantine E, Prando C, Giliani S, Israelsson E, Xu Z, et al. Immunodeficiency, autoinflammation and amylopectinosis in humans with inherited HOIL-1 and LUBAC deficiency. *Nat Immunol* 2012;13:1178–86.
33. Boisson B, Laplantine E, Dobbs K, Cobat A, Tarantino N, Hazen M, et al. Human HOIP and LUBAC deficiency underlies autoinflammation, immunodeficiency, amylopectinosis, and lymphangiectasia. *J Exp Med* 2015;212:939–51.
34. Zak DE, Schmitz F, Gold ES, Diercks AH, Peschon JJ, Valvo JS, et al. Systems analysis identifies an essential role for SHANK-associated RH domain-interacting protein (SHARPIN) in

- macrophage Toll-like receptor 2 (TLR2) responses. *Proc Natl Acad Sci U S A* 2011;108:11536–41.
35. Peltzer N, Rieser E, Taraborrelli L, Draber P, Darding M, et al. HOIP deficiency causes embryonic lethality by aberrant TNFR1-mediated endothelial cell death. *Cell Rep*. 2014 Oct 9;9(1):153–65.
36. Elton L, Carpentier I, Verhelst K, Staal J, Beyaert R, et al. The multifaceted role of the E3 ubiquitin ligase HOIL-1: beyond linear ubiquitination. *Immunol Rev*. 2015 Jul;266(1):208–21. Review.
37. HogenEsch H, Boggess D, Sundberg JP. Changes in keratin and filaggrin expression in the skin of chronic proliferative dermatitis (cpdm) mutant mice. *Pathobiol J Immunopathol Mol Cell Biol* 1999;67:45–50.
38. Seymour RE, Hasham MG, Cox GA, Shultz LD, Hogenesch H, Roopenian DC, et al. Spontaneous mutations in the mouse Sharpin gene result in multiorgan inflammation, immune system dysregulation and dermatitis. *Genes Immun* 2007;8:416–21.
39. Zhou Q, Yu X, Demirkaya E, Deutch N, Stone D, Tsai WL, et al. Biallelic hypomorphic mutations in a linear deubiquitinase define otulipenia, an early-onset autoinflammatory disease. *Proc Natl Acad Sci U S A*. 2016 Sep 6;113(36):10127–32.
40. Lin H-K, Fu L-S. Concurrence of Incontinentia Pigmenti and Behçet's Disease. *J Chin Med Assoc JCMA* 2010;73:275–8.
41. Juan C-K, Shen J-L, Yang C-S, Liu K-L, Chen Y-J. Flare-up of incontinentia pigmenti in association with Behçet disease. *JDDG J Dtsch Dermatol Ges* 2015;13:154–6.
42. Takada H, Nomura A, Ishimura M, Ichiyama M, Ohga S, Hara T. NEMO mutation as a cause of familial occurrence of Behçet's disease in female patients. *Clin Genet* 2010;78:575–9.
43. Ørstavik KH, Kristiansen M, Knudsen GP, Storhaug K, Vege A, Eiklid K, et al. Novel splicing mutation in the NEMO (IKK-gamma) gene with severe immunodeficiency and heterogeneity of X-chromosome inactivation. *Am J Med Genet A* 2006;140:31–9.
44. Wang Z, Potter CS, Sundberg JP, Hogenesch H. SHARPIN is a key regulator of immune and inflammatory responses. *J Cell Mol Med* 2012;16:2271–9.
45. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. *Am J Hum Genet* 1992;51:1229–39.
46. Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. *Science* 1991;252:1162–4.
47. McDonnell AV, Jiang T, Keating AE, Berger B. Paircoil2: improved prediction of coiled coils from sequence. *Bioinforma Oxf Engl* 2006;22:356–8.
48. Walshaw J, Shipway JM, Woolfson DN. Guidelines for the assembly of novel coiled-coil structures: alpha-sheets and alpha-cylinders. *Biochem Soc Symp* 2001;111–23.
49. Wood CW, Bruning M, Ibarra AA, Bartlett GJ, Thomson AR, Sessions RB, et al. CCBuilder: an interactive web-based tool for building, designing and assessing coiled-coil protein assemblies. *Bioinforma Oxf Engl* 2014;30:3029–35.
50. Roy A, Xu D, Poisson J, Zhang Y. A protocol for computer-based protein structure and function prediction. *J Vis Exp JoVE* 2011;e3259.

51. Rushe M, Silvian L, Bixler S, Chen LL, Cheung A, Bowes S, et al. Structure of a NEMO/IKK-associating domain reveals architecture of the interaction site. *Struct Lond Engl* 1993 2008;16:798–808.

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## Conflict of interest statements

Conflict of interest: none.

## Figure legends

### Figure 1: Identification of a mutation in the *IKBKG/NEMO* gene in a male fetus with IP (III.4).

**A.** Pedigree of family A, showing the segregation of microsatellite markers flanking the *IKBKG* gene. The black haplotype corresponds to three polymorphic markers surrounding the *IKBKG* gene and spanning a 4 Mb region, that segregates with IP in the family. X inactivation status in IP females: R: random X inactivation, S: skewed X-inactivation.

**B.** DNA sequence electropherograms around the exon 4 donor splice site of *IKBKG* in a male fetus with IP (family A, III.4) carrying the c.518+2T>G mutation, and in a control individual. Note a peak at the same position, corresponding to the pseudogene.

**C.** An electropherogram of the RT-PCR products, showing the full-length *IKBKG* cDNA (1200 bp) in a healthy individual and the abnormal band (around 900 bp) in the male fetus with IP (family A, III.4).

**D.** cDNA sequence electropherograms showing the skipping of *IKBKG* exons 4-6 in fibroblasts from the male fetus with IP, compared with control fibroblasts.

**E.** Immunoblot assays showing the wild-type NEMO protein in two fetal controls fibroblasts (42 kDa) and a smaller band (32 kDa) in the affected male fetus with IP carrying the c.518+2T>G mutation (p. DelQ134\_R256).

**Figure 2: Functional organization domains and structural models of the wild type and *IKBKG* mutant (Del 134-256).**

**A.** The deletion mutant (Del 134-256) consists of a mini-NEMO protein devoid of part of the CC1 coiled-coil and of the HLX2 helical domains as indicated. It contains all essential domains theoretically required for producing a functional NEMO protein. All NEMO structures solved to date are depicted in bright colors, whereas the modeled ones are in grey.

**B.** The two Ub-binding domains (the ZF domain and the NEMO-optineurin-ABIN domain, NOA, also referred to as UBAN or NUB) are shown as complexes with linear and K63 di-Ub chains, respectively. The figure shows the two C-terminal IKK- $\beta$  fragments (the NEMO binding domain, NBD, in light orange) interacting with NEMO kinase binding domain (KBD, in blue) and the binding site (NFZ1 BD) for the LUBAC subunit HOIP in the CC2 coiled-coil domain. The deletion introduces a novel junction between Q133 (CC1) and G257 (CC2-LZ), which causes a discontinuity in the regular heptad repeats in the CC1 and CC2-LZ domains. Furthermore, the deletion shortens the superhelical coiled-coil structure and thus changes the orientation of the NOA Ub-binding domain relative to the IKK- $\beta$  binding site.

**Figure 3: The NF- $\kappa$ B signaling pathway is impaired in patient cells carrying the *IKBKG* mutation.**

**A.** Immunoblot assays showing the normal degradation of I $\kappa$ B $\alpha$  proteins in control fibroblasts, as evidenced by the disappearance of the I $\kappa$ B $\alpha$  protein band after 10 or 20 minutes of stimulation with TNF- $\alpha$  or IL-1 $\beta$ . Degradation of I $\kappa$ B $\alpha$  was impaired in fibroblasts from the male fetus affected by IP (fetus III.4 and fetus NEMO  $-/-$  carrying the frequent *IKBKG* rearrangement Del exons 4-10 ).

**B.** ELISAs of levels of interleukin-8 and 6 (IL-8 and IL-6) in the supernatants of non-stimulated, TNF- $\alpha$ -stimulated and IL-1 $\beta$ -stimulated fibroblasts. There was almost no IL-8 and IL-6 expression in fibroblasts from the male fetus with IP (III.4) carrying the IVS4+2 T>G *IKBKG* mutation or in IP fibroblasts carrying the most frequent *IKBKG* gene mutation (deletion of exon 4 to 10, with full

inhibition of the NF- $\kappa$ B signaling pathways), relative to controls. Error bars: standard error of the mean (n=3); \*\*\* p<0.001.

**C.** Complementation assays in *Nemo*<sup>-/-</sup> MEFs after transfection with HA-tagged wild-type or truncated NEMO constructs and Ig $\kappa$ Luc and *Renilla* constructs. Luciferase was assayed after complemented cells had been stimulated with TNF- $\alpha$  and IL-1 $\beta$  for 24h. The truncated form of NEMO was unable to activate NF- $\kappa$ B in response to either TNF- $\alpha$  or IL-1 $\beta$ . Error bars: standard error of the mean (n=3); \*\* p<0.01.

**D.** IKK- $\beta$  or IKK- $\alpha$  plasmids were transfected into HEK293T cells with HA-tagged wild-type or truncated NEMO constructs. Immunoprecipitation was performed with anti-IKK- $\alpha$  or anti-IKK- $\beta$  antibodies. Immunoblotting analysis was then performed with an anti-HA antibody. The HA-tagged truncated NEMO interacted with IKK- $\alpha$  and IKK- $\beta$ .

**E.** TRAF6, TRAF2 or RIP1 plasmids were transfected into HEK293T cells with HA-tagged wild-type or truncated NEMO constructs. Immunoprecipitation was performed with anti-TRAF6, anti-TRAF2 or anti-RIP1 antibodies. Immunoblotting was then performed with anti-HA antibody. The same experiments were performed with inversion of the antibodies (i.e. immunoprecipitation with anti-HA antibodies and immunoblotting with anti-TRAF6, anti-TRAF2 or anti-RIP1 antibodies). The HA-tagged truncated NEMO interacted with TRAF6, TRAF2 and RIP1.

**F.** HA-tagged wild-type or truncated NEMO constructs were transfected into HEK293T cells with an HA-Ub expression vector in the presence or absence of TRAF6. Immunoblotting with an anti-NEMO antibody revealed similar K63-linked polyubiquitination patterns for the wild-type and truncated forms. Immunoblotting with an anti-TRAF6 antibody showed that TRAF6 was auto-ubiquitinated (bottom of the figure).

**G.** HA-tagged and FLAG-tagged wild-type or truncated NEMO constructs were transfected into HEK293T cells. Immunoprecipitation was performed with an anti-FLAG antibody, and then immunoblotting was performed with an anti-HA antibody. Truncated NEMO forms formed fewer homo/heterodimers than wild-type NEMO did.

The data are representative of three independent experiments.

**Figure 4: Interactions between LUBAC complex sub-units and wild-type or truncated NEMO forms.**

**A.** V5-SHARPIN, V5-HOIP or FLAG-HOIL-1 constructs were transfected into HEK293T cells with HA-tagged wild-type or truncated NEMO constructs. Immunoprecipitation was performed with an anti-HA antibody, and the subsequent immunoblots were performed with anti-FLAG or anti-V5 antibodies. Wild-type NEMO interacted with all three LUBAC sub-units. The truncated NEMO protein interacted with HOIL-1 and HOIP but not with SHARPIN.

**B.** Analysis of the interaction between wild-type or truncated NEMO forms and LUBAC subunits using DuoLink PLAs in transfected HEK293T cells. Cell nuclei were stained with DAPI (in blue). Signals indicating interaction or close relative positions were detected with confocal microscopy and appear as fluorescent dots (in red). In the PLA, only interacting epitopes located no more than 10-30 nm away from each other are labelled. Wild-type NEMO interacted with SHARPIN and HOIL-1 but not with HOIP. The truncated forms of NEMO interacted with HOIL-1 but not with HOIP or SHARPIN. Scale bar: 10  $\mu$ m. The data are representative of three independent experiments.

**Figure 5: Impaired recruitment of NEMO and SHARPIN to punctate structures in cultured fibroblasts.**

Non-treated or IL-1 $\beta$ -stimulated cells were permeabilized with saponin prior to fixation and then immunolocalization with anti-NEMO (green) and anti-SHARPIN (red) antibodies. The merged image shows that the NEMO-containing structures colocalized with SHARPIN in control fibroblasts from a healthy control. Recruitment of NEMO and SHARPIN to punctuate structures is impaired in (i) cultured fibroblasts from a male fetus carrying the splice c.518+2T>G *IKBKG* mutation, (ii) IP fibroblasts carrying the frequent *IKBKG* rearrangement Del exons 4-10 (NEMO<sup>-/-</sup>) and (iii) recently characterized HOIL-1-deficient fibroblasts from non-IP patients. Nuclei were stained with DAPI. Scale bar: 10  $\mu$ m.

**B.** Quantification of NEMO and SHARPIN in punctuate structures, showing a significant reduction in both NEMO and SHARPIN expression in patient cells. Quantification of the number of NEMO- and SHARPIN-containing spots per cell was performed for at least 50 cells.

**C.** Immunoblot assays show abnormally low of SHARPIN, HOIP and HOIL-1 in a patient's fibroblasts (carrying the truncated NEMO), relative to control fibroblasts.

**D.** Q-RT-PCR analysis of *SHARPIN*, *HOIP* and *HOIL-1* showed similar mRNA levels in fibroblasts from two fetal controls and fetus III.4. Results were normalized to *PGK-1*.

**Figure 6: Linear ubiquitination is defective in the patient's fibroblasts.**

Non-treated or IL-1 $\beta$ -stimulated cells were permeabilized with saponin prior to fixation and then immunolocalization with anti-NEMO (green) and anti-linear Ub chains (red) antibodies. Merged staining images show that the NEMO-containing structures colocalized with linear Ub chains in control fibroblasts but not in (i) IP cells carrying the splice mutation c.518+2T>G, (ii) IP cells carrying the frequent *IKBKG* rearrangement del exons 4-10 (NEMO<sup>-/-</sup>) or (iii) recently characterized HOIL-1-deficient fibroblasts from non-IP patients.

**B.** Quantification of the number of NEMO- and linear Ub-containing spots per cell (performed on at least 50 cells).

**C.** HA-tagged wild-type or truncated NEMO constructs were transfected into HEK293T cells with an Ubiquitin KO expression vector in the presence or absence of LUBAC (V5-SHARPIN, V5-HOIP or FLAG-HOIL-1). Immunoprecipitation and immunoblotting with an anti-HA antibody revealed a linear ubiquitination pattern for the wild-type NEMO but not for the truncated form.

**Supplementary Figure 1: Coiled-coil sequences and structural models of the WT and mutant NEMO (Del 134-256).**

**A.** The respective amino acid sequences of the WT and the mutant (Del 134-256) are shown with the heptad positions (letters (abcded)<sub>n</sub> below the sequence). Only regions near the deleted domain in the mutant are shown. The solved structures of NEMO fragments (HLX2, PDB ID: 3CL3 and CC2-LZ, PDB ID: 2V4H) are indicated as cylinders above the sequence. The heptad phases predicted by the PAIRCOIL2 coiled-coil prediction algorithm or determined from the crystal structures with the

SOCKET program are identical, as indicated by the underlined heptad repeat. Sequence analysis revealed atypical coiled-coil discontinuities in the HLX2:CC2-LZ region of the WT and at the CC1:CC2-LZ junction in the mutant (phase shift  $ab\downarrow\downarrow\text{efg}$ , with a deletion of two residues indicated in bold type). Nevertheless, modeling studies revealed that this region (which is better tolerated in the WT than in the mutant) can form a continuous coiled-coil structure by changing the local structure of the  $\alpha$ -helix and the left-handed superhelix.

**B.** Two different views of the superposed WT and mutant structures (based on the  $C\alpha$  of the N-terminal kinase domain (KD). K63-di-Ub chains bound to NOA or ZF sites are depicted at the surface for the WT (in bright red) and the mutant (the red blade). The deletion of 123 residues in the mutant (residues 134-256) shortens the left-handed superhelix of the NEMO coiled coil structure and thus modifies the orientations of the NOA and ZF Ub-binding domains relative to the IKK- $\beta$  binding site. These differences in the spatial arrangement of the Ub-binding domains may also contribute to the production of inactive NEMO-IKK- complexes.

Figure 1

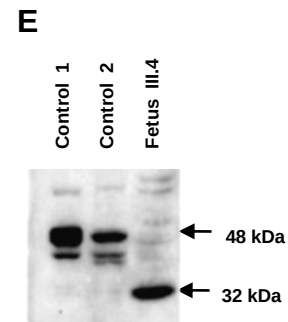
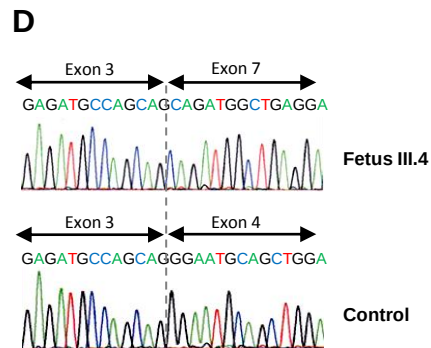
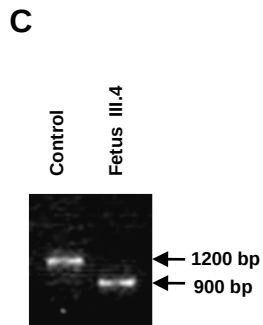
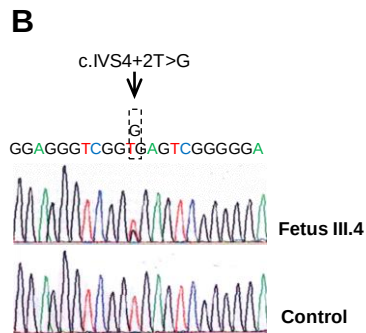
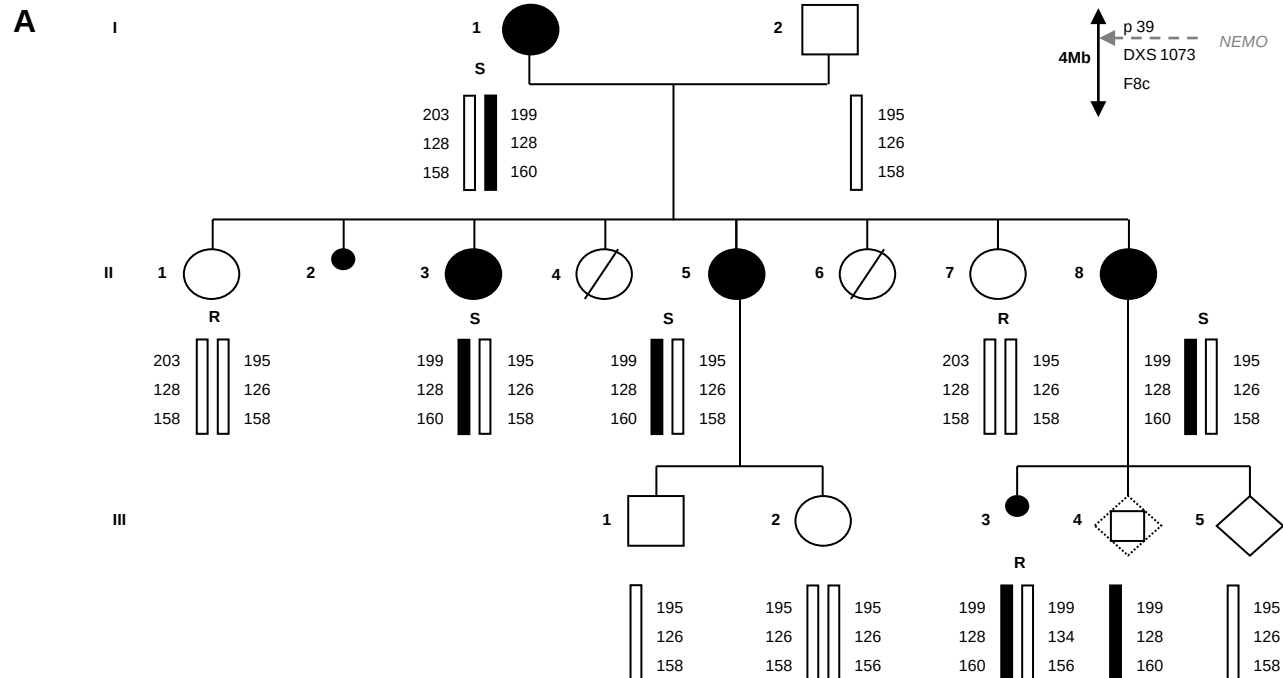




Figure 3

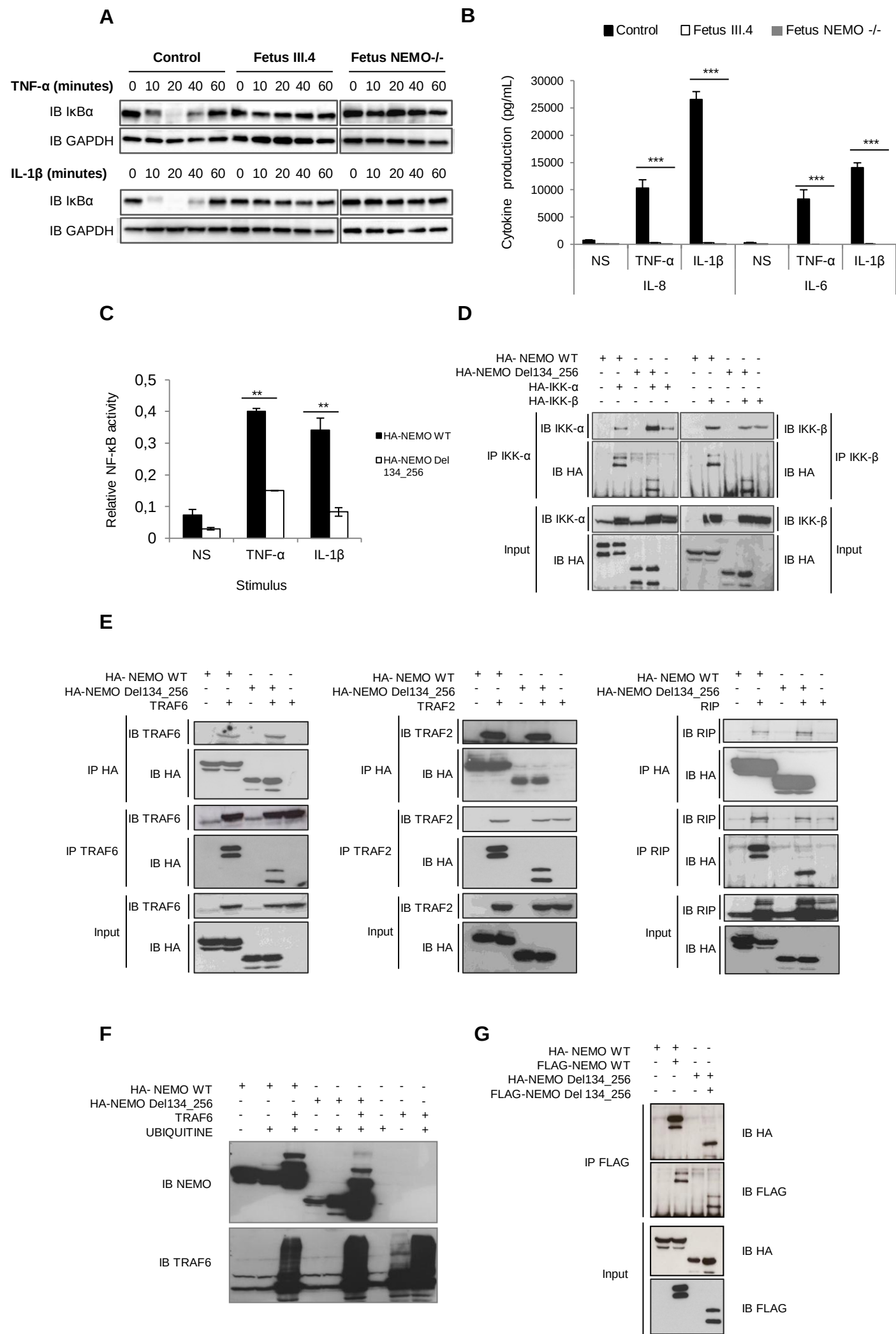
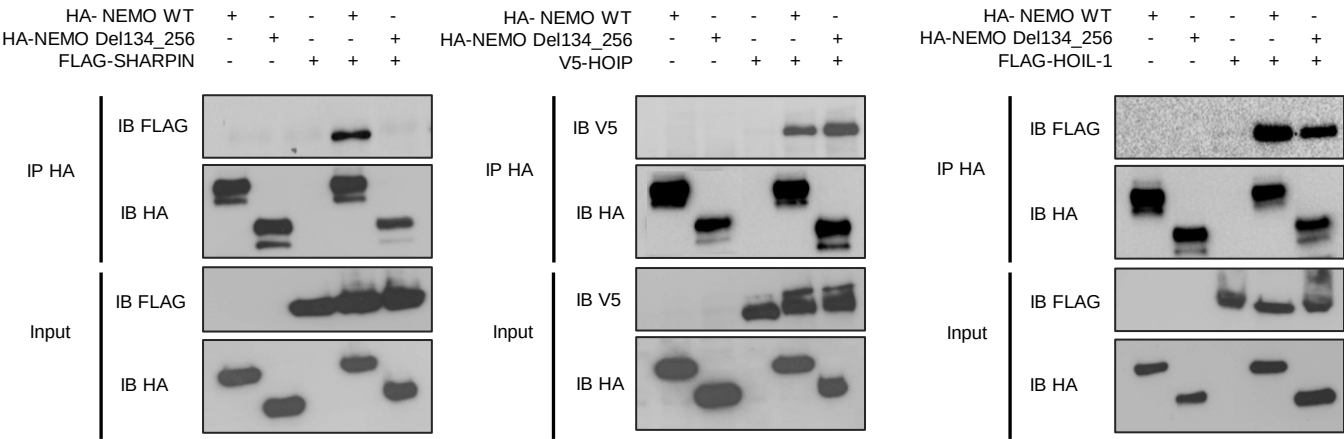


Figure 4

A



B

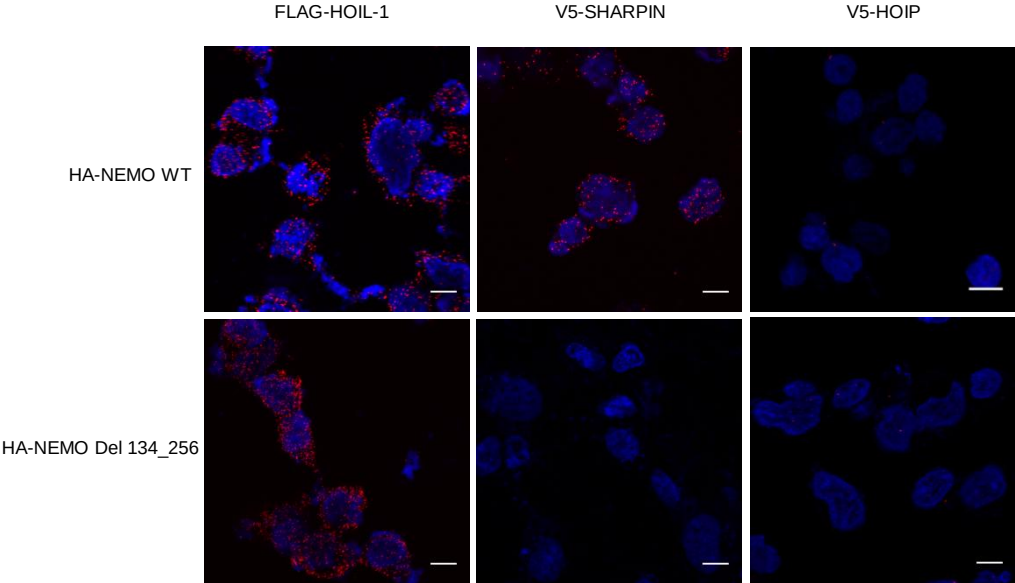


Figure 5

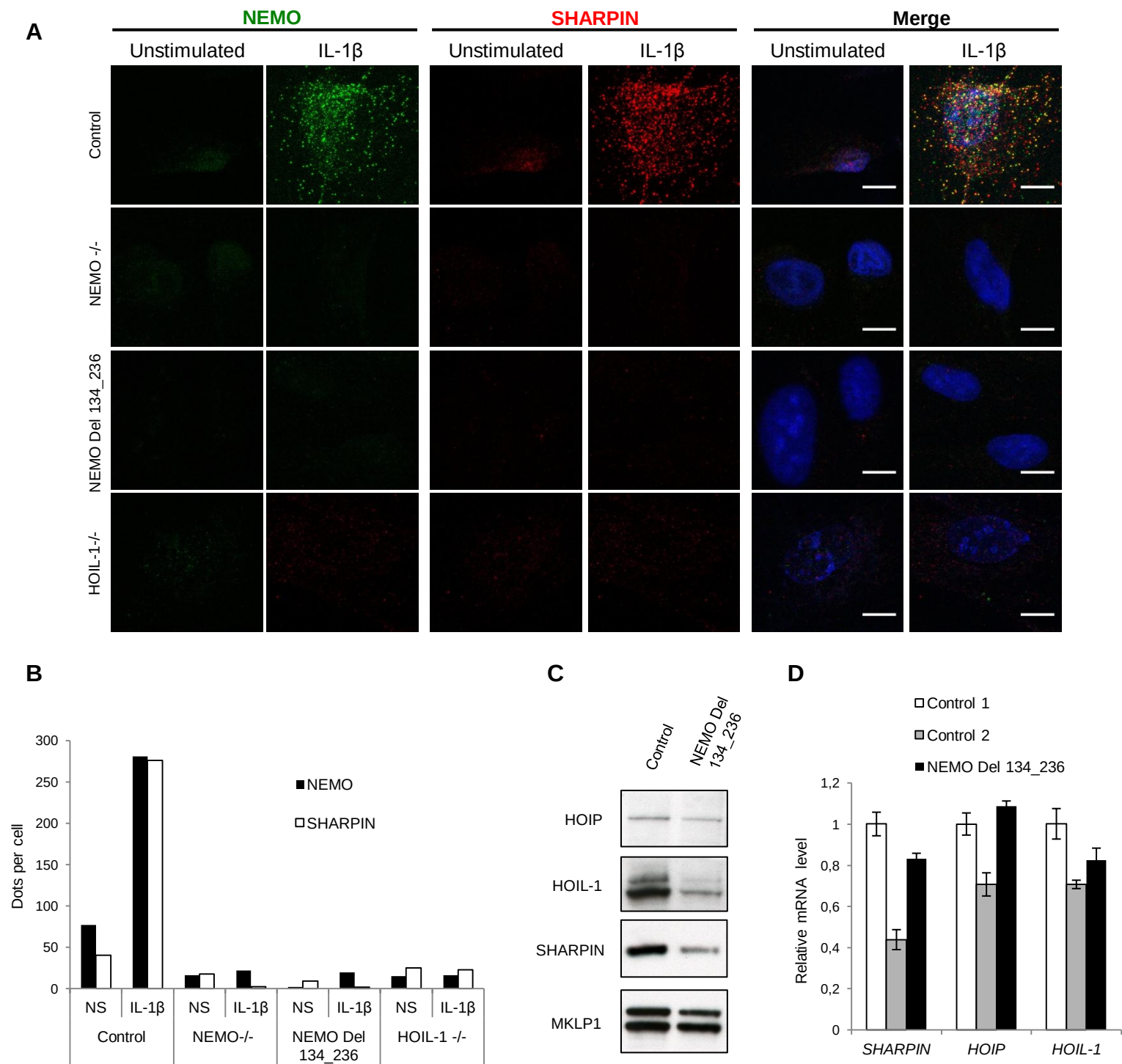
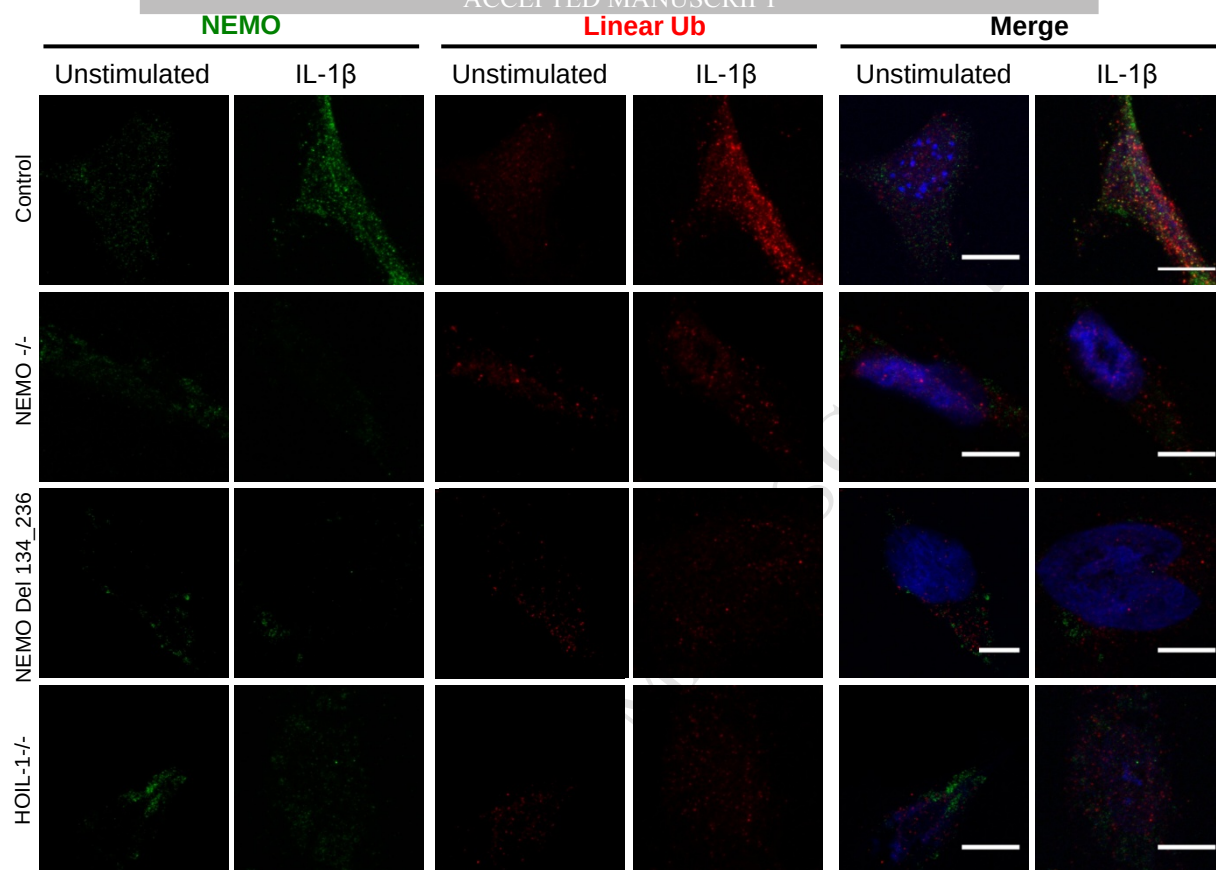
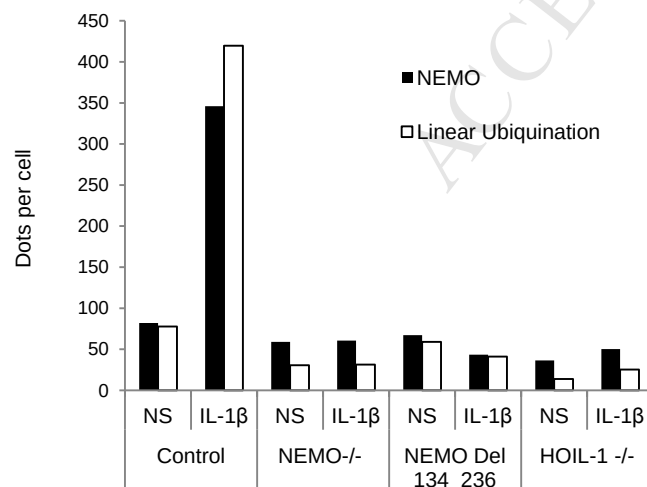


Figure 6

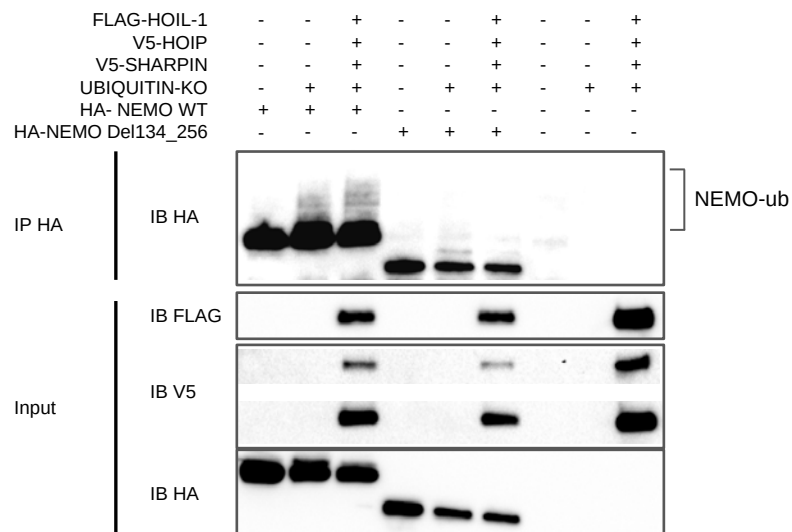
**A**



**B**

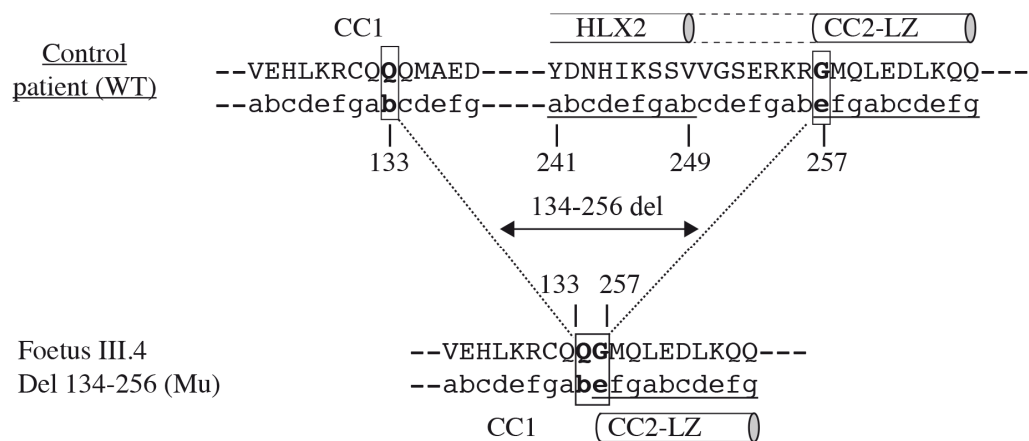


**C**



Supplementary Figure

A



B

