# Supporting Information

## Garcia-Cattaneo et al. 10.1073/pnas.1115091109

### SI Materials and Methods

**Cell Culture and Plasmids.** The hTERT-immortalized retinal pigment epithelial cell line (hTERT RPE-1) was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM:F-12 supplemented with 10% (vol/vol) FBS, penicillin/streptomycin, and sodium pyruvate. Hek293 (ATCC), and HEK-293 stably transfected with NF-κB Luciferase or IRSE reporter plasmid (HEK-293-luc and HEK-293-IRSE-luc, respectively, provided by S. Lebecque, University Claude Bernard Lyon1, Lyon, France) were cultured in DMEM supplemented with 10% (vol/vol) FBS, penicillin/streptomycin, and sodium pyruvate. Human monocyte–derived dendritic cells (MDDCs) were obtained as follows: PBMC were purified from blood from healthy donors, and monocytes were isolated by positive selection using CD14+ microbeads from Miltenyi. Monocytes were differentiated for 5 d to dendritic cells by culture in RPMI 1640 supplemented with 10% (vol/vol) FCS (Gibco), GM-CSF (0.1 μg/mL, final), and IL-4 (0.04μg/mL, final). hTLR3-4HA (WT-TLR3), cDNA encoding four repetitions of the hemmaglutinin (HA) epitope (5′TACCCATACGATGTTCCAGATTACGCT3′) was inserted at the 3′ end of TLR3 in pUNO-hTLR3 (Invivogen). 346-TLR3-4HA construct (346-TLR3) was generated from hTLR3-4HA by PCR cloning (sense sequence: 5′ATTGATGA-TTTTTCTTTTCAGTGGC3′). Constructs were transfected into cells by Gene Juice reagent (Merck) according to manufacturer's instructions. For stable transfection, cells were cultivated with 5 μg/mL Blasticidine S (Invivogen) for at least 2 wk.

**Reagents and Antibodies.** poly(I:C) (25 μg/mL, unless noted otherwise) (Invivogen), TNFα (5 ng/mL), IFNγ (100 units/mL), leupeptin (1 mM), z-FA-fmk (10 μM), concanamycin B (20 nM), cyclohexamide (10 μg/mL), dynasore (80 μM) (Sigma-Aldrich), and DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl-sulfate; Roche, accordingly to the manufacturer's instructions) were used for immunofluorescence and/or immunoblotting.

Rabbit anti-human NF-κB p65 (C-20) (Santa Cruz Biotechnology), mouse anti-human IRF3 (IRF35I218), and rabbit anti-human UNC93B (ab69497) (Abcam), Cy3 anti-rabbit and Alexa488 anti-mouse antibodies (Molecular Probes, Invitrogen) and 4',6-diamidino-2-phenylindole dilactate (DAPI) (Sigma-Aldrich) were used for immunofluorescence.

Rabbit anti-human cathepsin L (H-80), goat anti-human cathepsin H (*N*-18), goat anti-human cathepsin B (*N*-19) (Santa Cruz Biotechnology), rabbit anti-human AEP (D01P) (Abnova), mouse anti-actin (AC-40) (Abcam), rat anti-α tubulin (MCA77G) (Serotec), mouse anti-HA (HA.11) (Eurogentec), mouse anti-human TLR3 (kindly provided by S. Lebecque, University Claude Bernard Lyon1, Lyon, France) and HRP anti-rabbit, -goat, -mouse, and -rat (GE Healthcare) antibodies were used for immunoblotting.

All siRNA sequences were purchased from Qiagen, unless noted otherwise.

**Immunofluorescence.** RPE1 cells and MDDCs (after 5 d of differentiation) were seeded in 24-well plates with cover slips and treated with different reagents as indicated. After incubation, cells were fixed with either 4% (vol/vol) buffered paraformaldehyde solution (PFA, Sigma-Aldrich) for 15 min (for RPE1 cells) or 0,5% (vol/vol) PFA for 30 min (for MDDCs), quenched with 50 nM NH4Cl solution, and permeabilized with 0.5% (vol/vol) Triton X-100 in PBS for 5 min. Cells were stained with the indicated primary and secondary antibodies for 1 h, and cover slips mounted with fluoromount (SouthernBiotech) on slides for imaging. Image acquisition was made using a Nikon epifluorescence microscope at ×20 magnification.

**Automated Quantification of the NF-κB and IRF3 Nuclear Translocation.** Images were acquired using a Nikon epifluorescence microscope (Eclipse 90i Upright Microscope) at ×20 magnification for slides and ×10 for 96-well plates. Images were analyzed using algorithms based on ImageJ (National Institutes of Health), detecting the nuclear region by DAPI staining and defining a mask around the nucleus (nuclear) and a second mask five pixels larger (cytoplasmic) in NF-κB or IRF3 stainings. TLR3 activation was determined by the ratio of nuclear mean intensity versus cytoplasmic mean intensity after cell exposure to different treatments. For statistical purposes, experiments were analyzed by percentage of cells with NF-κB or IRF3 translocated into the nucleus. Frequencies of distribution were calculated from the ratio values given by the program. We considered as translocated the cells with ratio above 1 standard deviation of the control mean ratio (nontreated cells). In all experiments, at least 3 independent experiments were plotted and one-way ANOVA used for statistical analysis.

**Cytokine Secretion.** Cells were seeded 24 h before transfection with scrambled siRNA (allstar negative, Invitrogen), siRNA targeting TLR3 gene according to manufacturer's protocol, or treated with leupeptin or z-FA-fmk . After 18 h, cells were treated with poly (I:C) or TNFα at the indicated concentrations for 15 h, and assessed by IL-8 ELISA (eBioscience) according to the manufacturer's protocol.

**Immunoprecipitation and Immunoblot Analyses.** Cells exposed to different treatments as indicated were lysed in Nonidet P-40 modified buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 5mM $MgCl_2$, and 0.5% (vol/vol) Nonidet P-40) supplemented with complete protease inhibitor mixture (Roche). After incubation at 4 °C, lysates were cleared by centrifugation. For immunoprecipitations, lysates were precleared twice and incubated with protein G Sepharose (GE Healthcare) and anti-HA antibody overnight at 4 °C. Proteins were separated by SDS/PAGE and transferred to Immobilon PVDF membrane (Millipore). Deglycosylation kits EndoHf and PNGase F (New England BioLabs) were used accordingly to the manufacturer's instructions. Membranes were probed with the indicated antibodies and developed by West Dura Chemiluminescent Substrate (Thermo Fisher Scientific).

**RT-PCR UNC93B1.** Total RNA of the samples was extracted from cultured cells using RNA isolation kit from Macherey-Nagel (Nucleospin RNA II) accordingly to manufacturer's instructions. An aliquot (1 μg) of the RNA samples was then reverse transcribed (RT) using superscript VILO cDNA synthesys kit from Life Technologies. For quantitative PCR (qPCR), 1 μL of the RT products was used as template with hypoxanthine-guanine phosphoribosyl transferase-1 (HPRT1, Hs01003267_m1, Life Technologies) and designed primers for UNC93B1 (Hs00276771_m1, Life Technologies). qPCR was run on a LightCycler 480 (Roche) with the following cycling conditions: UNG actvation at 50 °C for 2 min and polymerase activation at 95 °C for 2 min and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The expression levels of UNC93B1 were quantified following the manufacturer's instructions.

**RNAi Transfection and Cathepsin Protease Library Screen.** Cells were transfected with four siRNAs targeting the Cathepsin protease

family and Asparagine Endo-Peptidase genes in a single siRNA format. Cells were treated in triplicate according to the manufacturer's protocol. Briefly, cells were seeded at a density of 2500 cells/well 24 h before RNAi. After 48 h, cells were treated with poly(I:C) (5 μg/mL) for 75 min. Prevalidated control siRNAs targeting TLR3, NF-κB, and TNF-receptor 1 (TNFR1) mRNAs were included into the screen procedure. Luciferase (GL2) and a lethal KIF11 (EG5) siRNAs were included as negative and positive transfection controls, respectively. Decreased cell viability in the lethal positive control wells served as an indicator of successful transfection for each plate.

**Screen Fluorescent Image Acquisition and Analysis.** Images of cells in 96-well plates were acquired in four fields per well using an IN Cell1000 high-content imager (GE Healthcare) using ×10 objective. Image analysis was performed using the IN Cell analyzer workstation 3.6 software that created nuclear outline masks in DAPI channel images, which were subsequently transposed onto the matched A488 channel exposures for that field of view. The mean intensity of nuclear NF-κB staining within previously defined DAPI masks was then quantified. The screening data were normalized using the standard z-score method by correcting the raw data for plate row variation and then normalizing and pooling data from all assay plates. The assumption is that the majority of the siRNAs are nonhits and the null distribution is normal (1). The criteria for identification of potential hits were z-score values of less than −3, which corresponded to a $P$ value of 0.05, in all of the three screens performed. This cutoff was chosen because of the relatively small size and focused nature of the screen.

1. Birmingham A et al. (2009) Statistical methods for analysis of high-throughput RNA interference screens. *Nat Methods* 6:569–575.

**Tandem Mass Spectrometry.** HEK-293 cells stably transfected with TLR3-4HA construct were used to immunoprecipitate TLR3. Lysates were incubated with μMACS Anti-HA column (Miltenyi Biotec). TLR3-positive bands were detected by SDS/PAGE, excised, trypsin digested, and subjected to tandem mass spectrometry in the Mass Spectrometry and Proteomics platform of the Curie Institute.

**NF-κB and IRSE Luciferase Reporter Assay.** HEK-293-luc and HEK-293-IRSE-luc cells stably transfected with the indicated TLR3 construct were plated in 96-well plates for 1 d before exposure to poly (I:C) (5 μg/mL) or TNFα as indicated. Luminescence measure was made after 10 min of cells incubation with Steady-Glo Luciferase Assay System (Promega) accordingly to the manufacturer's protocol.

**Cell Viability.** Cells were seeded in 96-well plates overnight before treatement with leupeptin or z-FA-fmk at the indicated concentration for 16 h. Luminescence measure was made after 10 min of cell incubation with Cell titer-Glo Luminescent Assay (Promega) accordingly to the manufacturer's protocol.

**Statistical Analysis.** For analyses of pooled data expressed as percentage of translocated cells for NF-κB or IRF3, luciferase reporter assays, and IL-8 expression one-way ANOVA was used. Differences between different treatments or time points were determined as significant using the Tukey honestly significant difference (HSD) intervals as follows: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Histograms were plotted as mean of at least three independent experiments ± SD. In all experiments using RPE1 cells, at least 100 cells per treatment were assessed, and for MDDCs at least 60 cells per experiment were assessed.

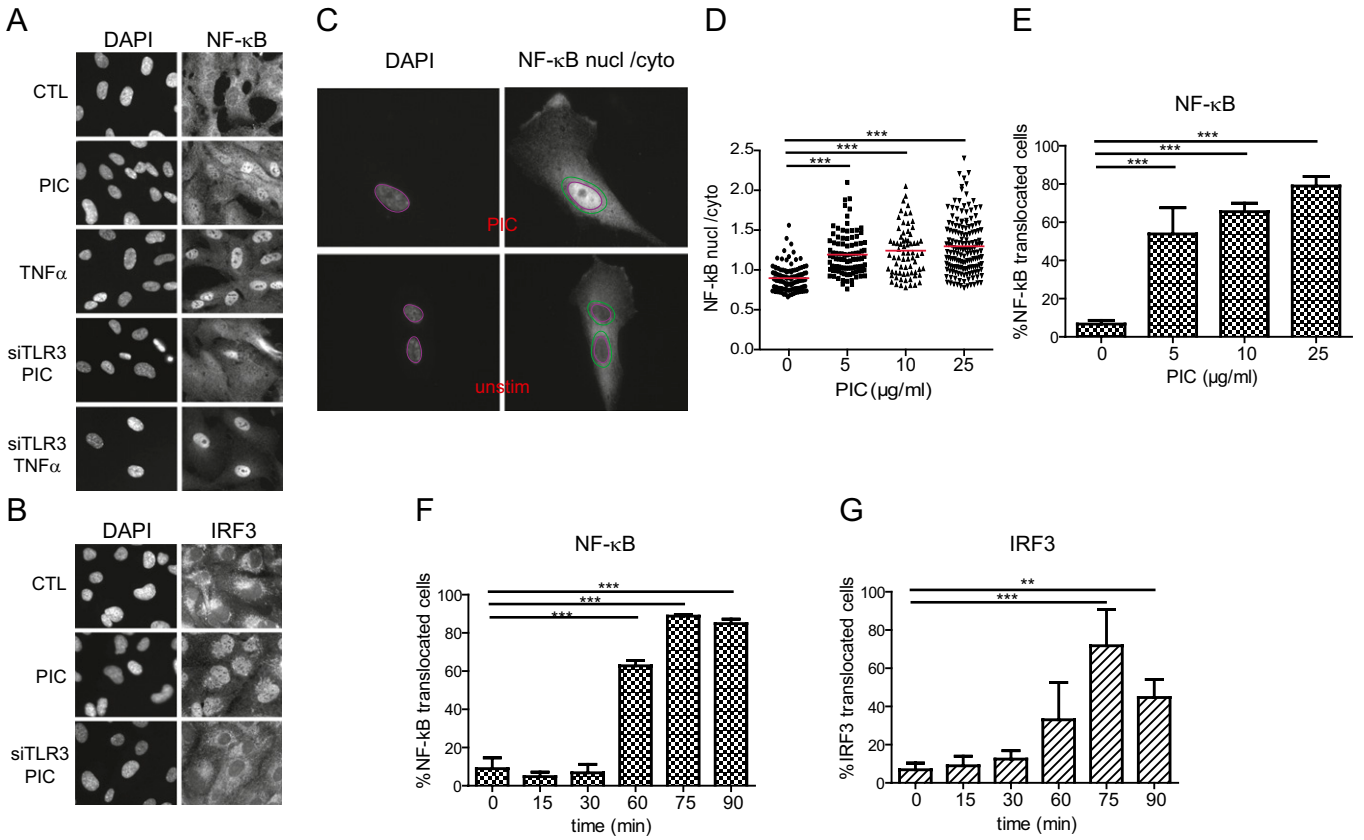


**Fig. S1.** TLR3-dependent responses of RPE1 cells to poly(I:C). (*A* and *B*) Translocation of NF-κB (*A*) and IRF3 (*B*) into the nucleus. RPE1 cells transfected or not transfected with siRNA specific for TLR3 were incubated with poly(I:C) (PIC) for 75 min, TNFα for 30 min or unstimulated (CTL), stained for NF-κB (A) or IRF3 (B) and assessed by epifluorescence microscopy. Nuclei were stained with DAPI. (*C*) Example of the Image J algorithm used to quantify NF-κB or IRF3 translocation to the nucleus. Images were analyzed detecting the nuclear region by DAPI staining and defining a mask around the nucleus (nuclear) and a second mask five pixels bigger (cytoplasmic) in NF-κB or IRF3 staining and the ratio of nuclear mean intensity versus cytoplasmic mean intensity after cell exposure to different treatments were calculated. Unstim, unstimulated cells. (*D* and *E*) Analysis of TLR3 activation in RPE1 cells for NF-κB pathway after 75 min of poly(I:C) (PIC) at the indicated concentration, represented as the ratio of nuclear mean intensity versus cytoplasmic mean intensity of one representative experiment in (*D*) (each dot represents one cell), or in (*E*) as % of cells with NF-κB translocated to the nucleus of three independent experiments. (*F* and *G*) Analysis of TLR3 activation in RPE1 cells exposed to 25 μg/mL poly(I:C) for the indicated time, represented as percentage of cells with NF-κB (*F*) or IRF3 (*G*) translocated to the nucleus. Data are from three independent experiments. One-way ANOVA with Tukey HSD, $^{**}P < 0.01$ or $^{***}P < 0.001$.

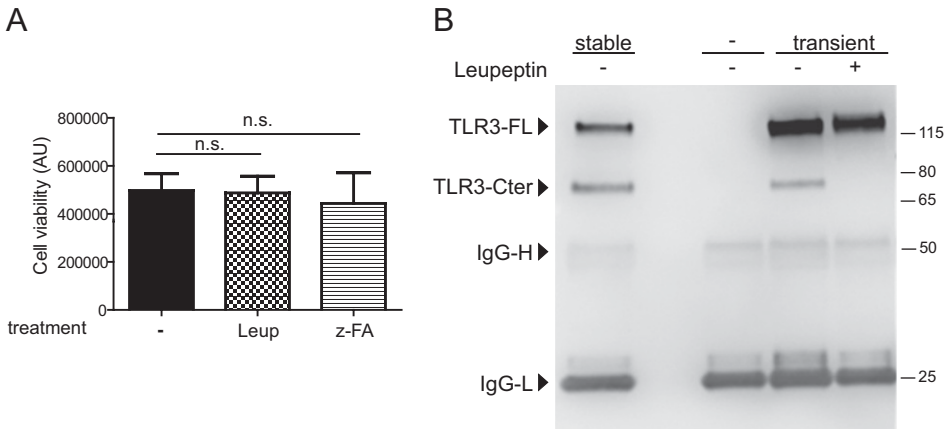


**Fig. S2.** TLR3 is cleaved by leupeptin-sensitive proteolytic activity. (*A*) Cells treated with leupeptin or z-FA-fmk for 18 h before stimulation with poly(I:C) from three independent experiments were assessed for viability. (*B*) Immunoblot analysis of HA-specific immunoprecipitations performed with HEK-293 cells stably or transiently transfected with TLR3-4HA, and exposed or not exposed to leupeptin for 26 h. Immunoblots were revealed with a HA-specific antibody. Data are representative of three experiments. One-way ANOVA with Tukey HSD, n.s., $P > 0.05$.

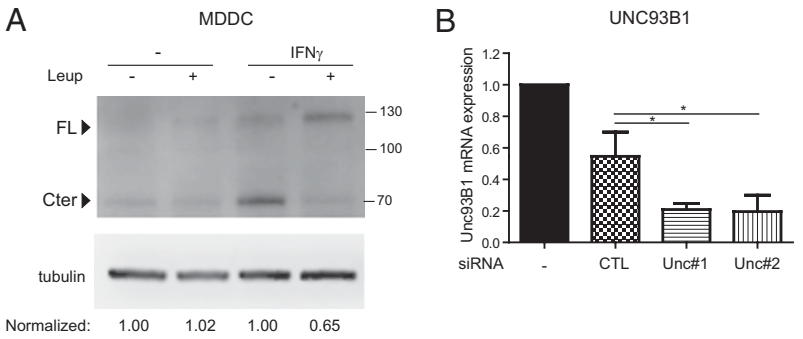


**Fig. S3.** An UNC93B1-dependent pool of cleaved TLR3 is present in endosomes ready to signal. (*A*) Immunoblot analysis of endogenous TLR3 expression. Prior to lysis, MDDCs were treated with leupeptin or not in presence or absence of IFNγ for 18 h. FL, TLR3 full-length; Cter, TLR3 C-terminal 70-kDa fragment. Bands corresponding to the full-length and the C-terminal fragment were quantified, and the percentage of the 70-kDa TLR3 C-terminal form over the full-length form is shown for each condition, and normalized within the treatment group. Tubulin is shown as a control for protein loading. Data are representative of three experiments. (*B*) RPE1 cells were transfected with UNC93B1 (Unc) or scrambled (CTL) siRNAs for 3 d and assessed by UNC93B1 RT-PCR. Data from four independent experiments.

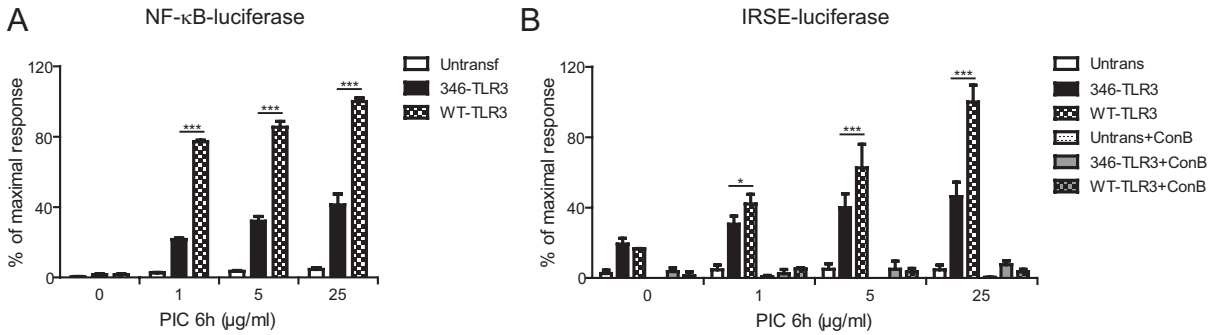


**Fig. S4.** A truncated mutant of TLR3 can transduce signal in response to poly(I:C). (*A*) Stably transfected HEK-293-luc cells were assessed by NF-κB luciferase reporter assay after 6 h exposure to poly(I:C). (*B*) Stably transfected HEK-293-IRSE luc cells were assessed by IRSE luciferase reporter assay after 6 h exposure to poly(I:C) before pretreatement or no pretreatment with ConB for 30 min. 346-TLR3, 346-TLR3-4HA; +ConB, cells pretreated with ConB; untransf, untransfected cells; WT-TLR3, WT-TLR3-4HA;. Data are from three independent experiments. One-way ANOVA, Tukey HSD, $*P < 0.05$ or $***P < 0.001$.

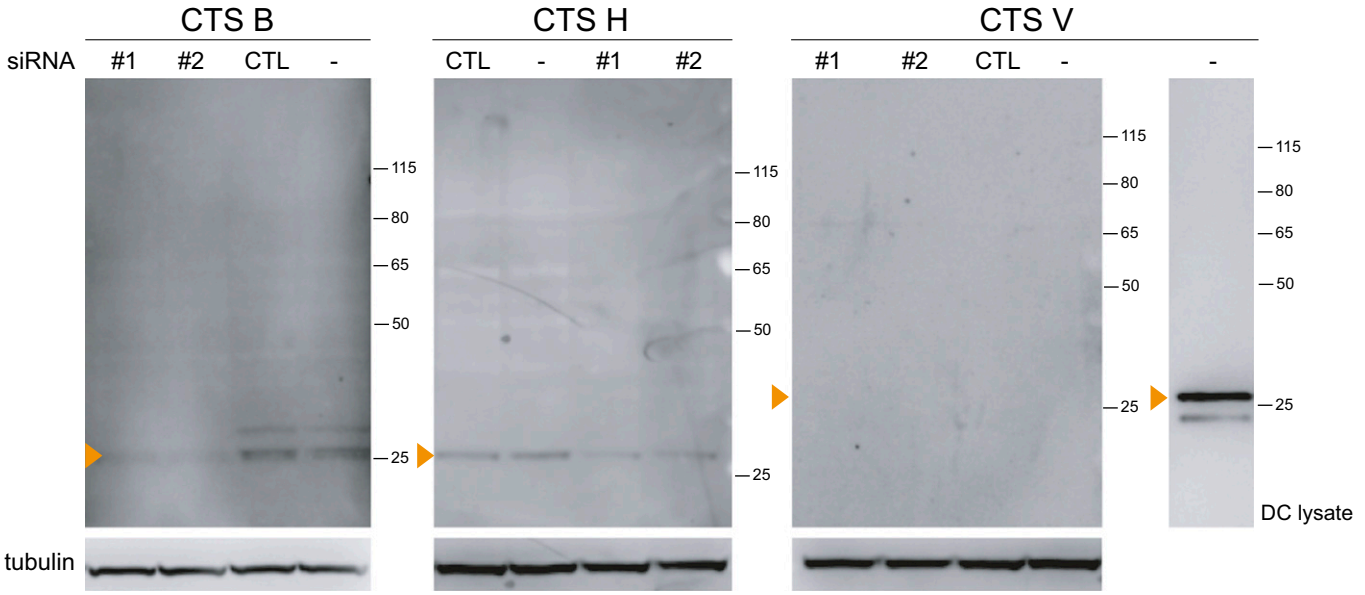


**Fig. S5.** Analysis of cathepsins B, H, and V expression. Immunoblot analysis of endogenous cathepsins present in the indicated RPE1 cell lysates. Cells were transfected with the indicated siRNA for 3 days before lyses. Tubulin is shown as a control for protein loading. DC lysate is shown as a control for cathepsin V antibody staining. Orange arrow represents the expected molecular weight of each cathepsin. CTL, scrambled siRNA; CTS, cathepsin. Data are representative of three independent experiments.

**Table S1. Cleavage of TLR3 by cathepsins B and H is required for signaling**

| Gene name | No. of siRNAs | siRNA 1 | siRNA 2 | siRNA 3 | siRNA 4 |
|---|---|---|---|---|---|
| *CTSA* | 0 | −2.16 | −0.07 | 0.67 | 2.16 |
| *CTSB* | 1 | 0.54 | 1.34 | 1.15 | −5.05 |
| *CTSC* | 0 | −0.45 | 2.09 | 1.39 | 0.33 |
| *CTSD* | 0 | −2.66 | −2.3 | −0.37 | 1.01 |
| *CTSE* | 0 | −2.04 | 1.26 | 0.72 | 0.42 |
| *CTSF* | 0 | 1.28 | 0.21 | 1.19 | −2.54 |
| *CTSG* | 0 | −0.45 | 1.07 | 0.19 | 0.19 |
| *CTSH* | 2 | −3.99 | 1.69 | −3.92 | −0.47 |
| *CTSK* | 0 | 1.75 | 1.02 | 0.84 | 1.43 |
| *CTSL* | 0 | 0.98 | 1.18 | 1.24 | 0.48 |
| *CTSO* | 0 | 1.25 | 0.88 | 0.04 | −0.7 |
| *CTSS* | 0 | 1.13 | 0.42 | −0.21 | −1.58 |
| *CTSV* | 1 | −3.28 | 0.08 | 1.24 | −0.31 |
| *CTSW* | 0 | 0.3 | −1.44 | 0.18 | 1.49 |
| *CTSZ* | 0 | −0.84 | 0.61 | 1.17 | 0.3 |
| *AEP* | 0 | 0.5 | 0.73 | −0.15 | 1.13 |
| *TLR3* | 4 | −5.53 | −6.55 | −6.64 | −6.87 |
| *RELA* | 4 | −6.48 | −7.26 | −6.8 | −6.7 |
| *TNFR1* | 0 | −1.56 | 0.43 | 0.9 | 1.23 |

Analysis of TLR3 activation by NF-κB translocation after poly(I:C) stimulation of RPE1 cells previously transfected with siRNA specific for each of the 15 human cathepsins and AEP (four different siRNA/gene). Data are represented as mean value of the three independent experiments for each siRNA. A hit is considered (in yellow) when at least one of the four siRNA/gene had a Z factor inferior to −3 ($P < 0.05$) in 3 out of the three experiments. Positive controls are represented in orange.

**Table S2. Sequences of siRNA used for RNA interference assays**

| | Screening 1 | | | | Screening #2 | |
|---|---|---|---|---|---|---|
| Gene name | siRNA 1 | siRNA 2 | siRNA 3 | siRNA 4 | siRNA 1 | siRNA 2 |
| *CTSA* | TCCGGAGTACAAGAACAACAA | CCGAAGCATGAACTCCCAGTA | ATACCAGATCCTATTATATAA | CCGGCCCTGGTTAGTGAAGTA | | |
| *CTSB* | CAGGATCACTGTGGAATCGAA | CAGCATGATTCTTTAATAGAA | CAGGAGTGTACCAACACGTCA | TTCGGACTTCCTGCTCTACAA | GCTGGTCAACTATGTCAACAA | TTCGGACTTCCTGCTCTACAA |
| *CTSC* | TGAGATTGTGTTGAATGACTA | CTCGGTTATGGGACCACAAGA | ATGATCTGCATCAGTTGTAAA | CTGCAATGAAGCCCTGATGAA | | |
| *CTSD* | CTGGATCCACCACAAGTACAA | CAGCGACAAGTCCAGCACCTA | CCCAGAGGACTACACGCTCAA | CCCAGCTGACATGGAAATACA | | |
| *CTSE* | CCCAGTCACCAAGCAAGCTTA | CAGGAGGCATCCGTCCCTCAA | CTGGCAGATTGCACTGGATAA | CTGGTCGGACTCACAATGAAA | | |
| *CTSF* | TCCGCACTATCTACCTGAATA | CCTGTCCGTCTTTGTCAATAA | CACCTTTGTTGAATTGTGGTA | CCCAATCTCCGTGGCCATCAA | | |
| *CTSG* | CGGCATCGTCTCCTATGGAAA | CACAATATCCAGAGACGGGAA | CAGACGGAATCGAAACGTGAA | CACAGTGTTGCCAGAGCCTTA | | |
| *CTSH* | GAGGAAGATAAACGCCCACAA | ATCGGCTTTGTCAAGGATGTA | CGAGTATATCCTGTACAACAA | ATGGATGTCTAAGCACCGTAA | CGAGTATATCCTGTACAACAA | GAGGAAGATAAACGCCCACAA |
| *CTSK* | AGGGCTGATGCTGTACAGGTA | CCCGATGGAATAAATCTAGCA | CACGGGACTAGTTAGCTTTAA | CCGCAGTAATGACACCCTTTA | | |
| *CTSL* | TGAGTCCTTCCTGTTCTATAA | CAGAAGAATCCTGTAAGTACA | AAGGCGATGCACAACAGATTA | AAGATCCGAGTGTGATTTGAA | TGAGTCCTTCCTGTTCTATAA | CAGAAGAATCCTGTAAGTACA |
| *CTSL2* | TTCGTCCTTCCAGTTCTACAA | AAGACTCATTGCTTAATTCTA | CAGCAAGTATTGGCTCGTCAA | AAGGAGCAAATTCGAATAACA | | |
| *CTSO* | TGCAGTGAGCTGGCAAGATTA | TTCCGGGAAAGTCTTAATAGA | TCGGAAGGATTTCAAACACCA | CACAAGAATGGCCAACCTAAA | | |
| *CTSS* | CTCATACGATCTGGGCATGAA | CTGCCACATGTTCAAAGTACA | AGGAATCTAATTATATCGAAA | AAGGATATATTCGGATGGCAA | | |
| *CTSW* | CCCGTGTGCAGAAACCGGATA | CCCAGCATGGGCAGAGAAATA | CGCGTTCATAACTGTCCTCAA | CACCGTGACCATCAACATGAA | | |
| *CTSZ* | CAGATACAACCTTGCCATCGA | ATCGAGGAGCACTGTACATTT | TCGGATCAACATCAAGAGGAA | ACCGGAGGCATCTATGCCGAA | | |
| *AEP* | GACGTGGAAGATCTGACTAAA | CTGCCGGATAACATCAATGTT | CGCAATGGGATTCCTGACGAA | AAGGGTCATATTTGCTTTCTA | GACGTGGAAGATCTGACTAAA | CTGCCGGATAACATCAATGTT |
| *TLR3* | AAGAACTGGATATCTTTGCCA | CACATTAGATCTGTCTCATAA | CAGCACAATGAGCTATCTCAA | CTGGATATCTTTGCCAATTCA | | |
| *RELA* | ATGGAGTACCCTGAGGCTATA | CCGGATTGAGGAGAAACGTAA | AAGATCAATGGCTACACAGGA | CATGGATTCATTACAGCTTAA | | |
| *TNFR1* | AAGTGCCACAAAGGAACCTAC | CTGGAGCTGTTGGTGGGAATA | AGGAACCTACTTGTACAATGA | ACGGTGGAAGTCCAAGCTCTA | | |
| | UNC93B1 Screening | | | | | |
| *UNC93B1* | CTGCCCGACATCGACAGCAAA | CACCAACTACTGGGAGCGCTA | | | | |

Screening 1, corresponding to results shown in Table S1; screening 2, corresponding to results shown in Fig. 6; and UNC93B1 screening, corresponding to results shown in Fig. 3.