Supporting Information

Crohn's disease variants of Nod2 are stabilized by critical contact region of Hsp70

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I. Materials

Primers were purchased from MWG Operon. Restriction endonucleases were purchased from New England Biolabs. The pFastBacM30b vector was received from European Molecular Biology Laboratory. Antibiotics were purchased from Gold Biotechnology. Expression hosts were purchased from Agilent Technologies. Insect cells were purchased from Life Technologies (ThermoFisher Scientific). All other chemicals were purchased from Sigma-Aldrich, unless otherwise indicated. All cell lines used were obtained from American type culture collection (ATCC, Manassas, VA). All cell lines were cultured in DMEM, 10% FBS (Atlantic Biologicals), 2 mM L-glutamine, penicillin-streptomycin and grown in a humidified incubator at 37°C and 5% CO2. Rabbit Nod2 antiserum (HM2559) was provided by the Podolsky laboratory and was purified by protein A column. Antirabbit IgG HRP linked secondary antibodies (Cell Signalling Technology) were used for immunoblot. MDP was purchased from Bachem and reconstituted in water at a concentration of 10 mM. 6-amino MDP was prepared as previously described [1-2].

II. Protein Expression, Purification, and Characterization

Nod2 expression and purification – All Nod2 protein constructs (WT, R702W, G908R, and 1007fs) were expressed and purified from insect cells (Sf21) according to the following method. GST-tagged Nod2 was generated by cloning WT Nod2 and mutants R702W, G908R, and 1007fsinsC into the pFastBacM30b baculovirus expression vector (EMBL). Nod2 (WT, R702W, G908R, and 1007fs) was amplified from the pBKCMV vector using the Nod2 primers (Table S1) and inserted into the pFastBacM30b vector using restriction sites BamH1 and Not1. Constructs were confirmed by DNA sequencing (GENEWIZ, Inc). The recombinant vectors and bacmids used to initially transfect insect cells were generated, isolated, and analyzed per the manufacturer's instructions (Invitrogen Life Technologies). Briefly, overexpression of the desired proteins was carried out in Sf21 cells infected with 10% of the desired recombinant baculovirus (P4) and incubated on a shaker at room temperature for two days. Cells were harvested by centrifugation at 800 rpm for 10 minutes, and pellets were stored at -80°C until purification.

Table S1: Primer DNA sequences used for molecular cloning.

Construct	Primer
Nod2, forward	5'GTCAATGGATCCATGGGGGAAGAGGGTGG3'
Nod2, reverse	5' CAATGCGGCCGCTCAAAGCAAGAGTCTG3'
S-71, forward	5'CGCGGATCCATGGATTACAAGGATGACGACGATAAGCTGGAGACGGCC3'
S-71, reverse	5'TCGACTCGAGTCAGATCTGGGGCACGCCCCTGG3'
Hsp70, forward	5'CGCGGATCGCGATGGATTACAAGGATGACGACGATAAGATGGCCAAAGC CGCGGCG3'
Hsp70, reverse	5'TCGACTCGAGCTAATCTACCTCCTCAATGGTGGGGCCTGACCCAGACC3'
Hsp70, SBD, forward	5'CGCGGATCGCGATGGATTACAAGGATGACGACGATAAGAACGTGCAGGACCT GCTGCT3'
Hsp70, SBD, reverse	5'TCGACTCGAGCTAATCTACCTCCTCAATGGTGGGGCCTGACCCAGACC3'
Hsp70, K71S, forward	5'AACACCGTGTTTGACGCG**TCG**CGGCTGATTGGCCGCAAG3'
Hsp70, K71S Reverse	5'CTT GCGGCCAATCAGCCG**CGA**CGCGTCAAACACGGTGTT3'

Purifications were performed at 4°C. A pellet from a 50 mL culture was re-suspended in 1 mL of cold lysis buffer (50 mM TrisHCl, 150 mM NaCl, 10 mM EDTA, 1 mM DTT, 10% glycerol, and protease tablet (Promega); pH 7.4) and lysed by repeated passage through a 20 gauge needle (approximately 15 times) for 1.5 hours. Soluble lysate was isolated by centrifugation at 15,000 rpm for 15 min. The supernatant was applied to 1 mL of Glutathione Sepharose resin (GE Healthcare) on a gravity column equilibrated with TBS buffer (50 mM TrisHCl, 150 mM NaCl, and 1 mM DTT; pH 7.4) and allowed to incubate on the shaker for 3 hours. In order to remove nonspecifically bound protein, the system was washed using a step gradient of NaCl (300 and 600 mM) in TBS buffer, pH 7.4, and finally returned to TBS buffer, pH 7.4. Protein was eluted by incubating overnight with PreScission protease (cleaving the GST tag) in TBS. The eluate was collected and immediately analyzed on SDS-PAGE gels to assess purity and concentration

(Figure S2). Protein was confirmed by proteomic mass spectrometry (AB Sciex 4800 – MALDI TOF Analyzer). Purified protein was stored at -80°C with the addition of 15% glycerol.

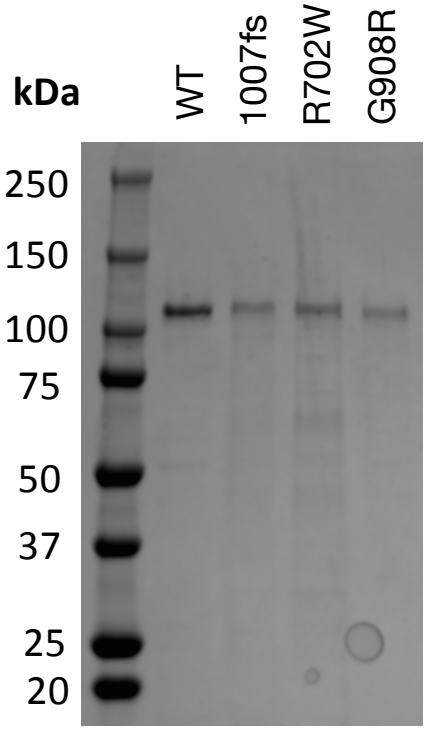


Figure S1. SDS-Page analysis of Nod2 protein constructs: GST tag was cleaved during purification. Expected MW of Nod2 is 115 kD. Expected MW of 1007fs is 111 kD.

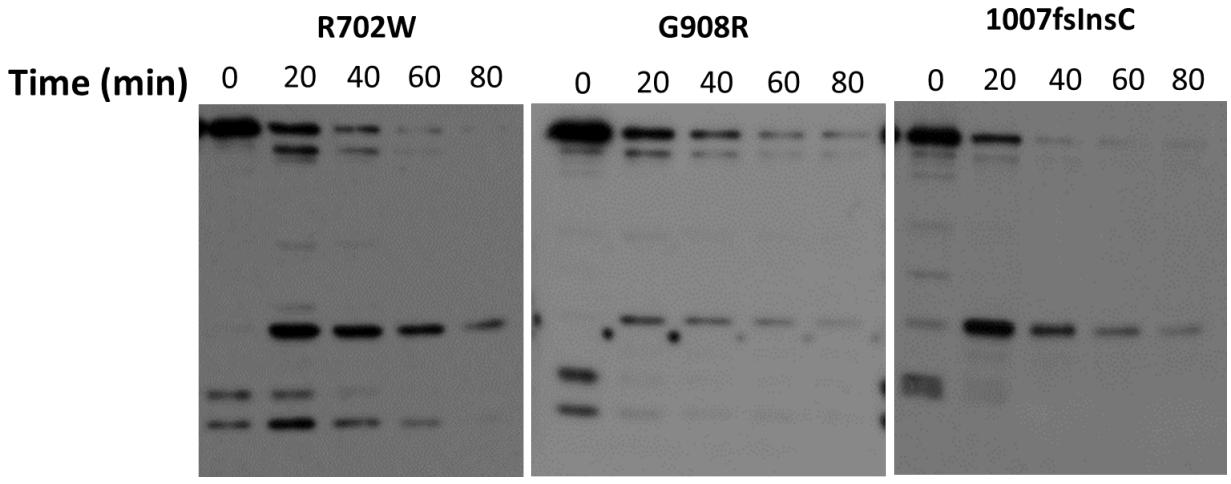


Figure S2. Limited proteolysis of CD mutant constructs: Purified Nod2 mutant constructs were treated with trypsin for 0 to 80 minutes at room temperature and analyzed by SDS-PAGE

followed by Western Blot analysis for NOD2 using NOD2 antibody. Wild type Nod2 limited proteolysis was previously reported[S2].

Hsp70 molecular cloning – HSP70 DNA was obtained from Addgene (pcDNA5/FRT/TO HIS HSPA1A). Full length and select domain cDNA fragments were amplified by PCR (primers in Table S1), inserted into the pGEX6P-1 vector using BamH1 and Xho1 restriction enzymes and transformed into DH5α competent cells. Constructs were confirmed by DNA sequencing (GENEWIZ, Inc). The plasmids were purified and transformed into BL21-CodonPlus(DE3)-RIPL competent cells (Agilent Technologies) according to the manufactures instruction. Colonies were picked and cultured at 37°C under appropriate antibiotics until the OD600 reached an absorbance of 0.6. Cultures were induced with 1mM IPTG overnight at 18°C. Growths were spun down in the centrifuge at 8000 rpm for 10 min. The supernatant was discarded and the pellets were stored at -80°C until purification.

Site directed mutagenesis—The K71S mutation was introduced in Hsp70 using GeneArt® Site-Directed Mutagenesis PLUS System kit on the Hsp70/pGEX plasmid per the manufacturer's instructions. Primer for Hsp70 found in Table S1. The presence of mutation in Hsp70 was verified via sequencing. Hsp70(K71S)/pBKCMV vector was constructed by digesting Hsp70(K71S) from Hsp70(K71S)/pGEX vector using BamH1 and Xho1 enzymes. The ligated products were transformed into competent cells. The presence of the insert within the construct was confirmed by DNA sequencing (GENEWIZ, Inc). The mutant Hsp70(K71S) was expressed and purified as described for the wild type Hsp70.

Hsp70 purification – The following were all done on ice or at 4°C: The pellet generated from 1 L of growth was resuspended in 30 mL of Buffer A (50 mM Tris buffer, pH 7.0, containing 150 mM NaCl, 1 mM EDTA, 1 mM DTT, and an EDTA-free protease tablet (Roche)). The cells were lysis by sonication. Lysed cells were clarified at 15000 rpm for 20 min and applied to a gravity column containing 5 mL of Glutathione Sepharose 4 Fast Flow resin (GE LifeSciences) equilibrated with Buffer A for 2 h. The flow-through was removed and the beads were washed with wash buffer (50 mM Tris buffer, pH 7.0, containing 500 mM NaCl, 1 mM EDTA). The column was equilibrated with 10 mL of 50 mM Tris buffer, pH 7.0 containing 1 mM DTT, 150 mM NaCl, and 1 mM EDTA and bound fusion protein was digested via on-column cleavage with the addition of 100 units of PreScission protease. Digestion was typically allowed to

proceed overnight at 4°C. After digestion, cleaved Hsp70 was eluted from the column. The eluate was collected and protein concentration was determined by Bradford assay using BioRad Protein Assay Dye Reagent Concentrate according to the manufacturer's instruction. Briefly, 1 mL of 1X dye was mixed with 2 µL of protein lysates and the absorbance was measured at 595nm. Absorbance values were converted to concentration using the standard curve obtained using the known concentrations of bovine serum albumin. Protein samples were prepared by adding a 15 µL aliquot to 5X loading buffer (250 mM TrisHCl pH 6.8, 10% SDS, 30% glycerol 0.02% bromophenol blue, 5% BME) and boiled for 5 min. The samples were electrophoresed in 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (Figure S3). The gel was stained using coomassie brilliant blue stain.

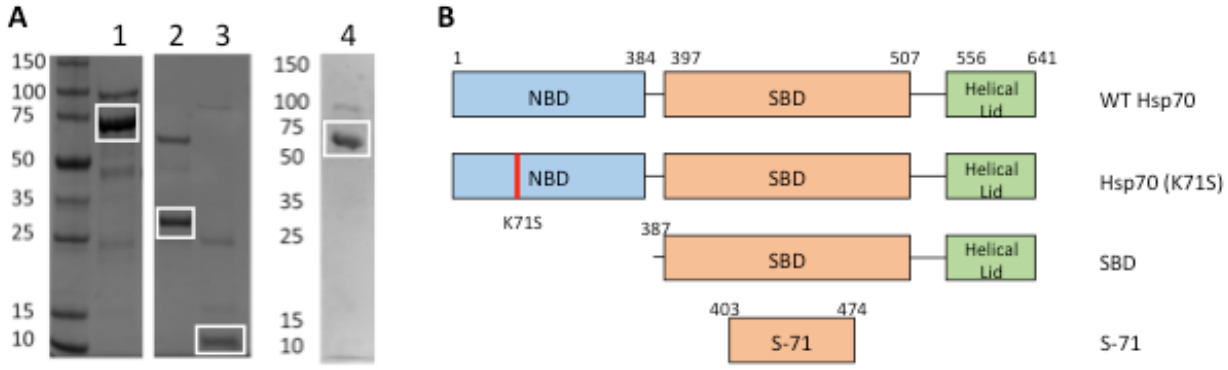


Figure S3. Hsp70 protein constructs : A: SDS-Page analysis of Hsp70 protein constructs. Hsp70 and its domains purified from *E.coli* cells. Ladder followed by lanes (1) Hsp70 (2) Substrate-binding domain (residues 387 to 641) (3) S-71 (residues 403 to 474) and (4) Hsp70 (K71S). Boxed area in each lane is the protein of interest. The additional band at ~55 kD in lane 3 corresponds to uncleaved GST construct. Expected MW of: Hsp70 is ~70 kD, substrate-binding domain is ~ 25 kD, Hsp70(K71S) is ~ 70 kD and S-71 is ~7 kD. B: Schematic representation of the engineered human Hsp70 variants used in this study

III. Surface Plasmon Resonance

Gold chip preparations – Gold chips (GE Healthcare Lifesciences) were stored at 4°C and handled only under laminar flow prior to mixed-SAM formation. Preparation of the Gold Thiol Chips (Mixed-SAMs) was carried out using previously reported methods[1, 3]. Ligands were immobilized using the Biacore 3000 instrument (GE Healthcare) according to previously reported methods[1, 3]. Briefly, chip surfaces were equilibrated with 1X PBS running buffer (prepared from 10X Lonza BioWhittaker without Ca^{2+} and Mg^{2+}, pH 7.4, filtered and degassed)

followed by transformation of the surface carboxylic acid groups into NHS esters by flowing a mixture of 0.05 M NHS and 0.20 M EDC over the surface for 7 minutes, after which the system was returned to 1X PBS running buffer for 1.5 minutes. Ligand solutions were applied to separate flow cells for seven minutes to form amide bond by displacement of the NHS esters. The system was returned to 1X PBS running buffer for 1.5 minutes and the surface was deactivated by washing with 1X PBS, pH 8.5. Following coupling the system was returned to 1X PBS running buffer for 23 minutes.

Equilibrium binding experiments – Purified Nod2 mutant constructs were assayed for the ability to bind MDP in an equilibrium binding analysis. Solutions (0, 0.65, 3.25, 6.5, 12.5, 25.0, 32.5, 65.0, 130, 260 nM) of purified Nod2 were prepared by dilution of the stocks into the SPR running buffer (10 mM MES, 150 mM NaCl, 3 mM EDTA, 0.005% P20, pH 6.5, filtered and degassed). Nod2 solutions were applied to the chip surfaces in triplicate at a flow rate of 3 uL min-1 for twenty minutes using the KINJECT command, washed with MBS running buffer for ten minutes, 0.5% (w/v) SDS for three minutes and the MBS running buffer for ten additional minutes. The binding of Nod2 to the chip was recorded in resonance units (RU) when the injection reached equilibrium. Sensorgrams for each mutant run are shown in Figure S4.

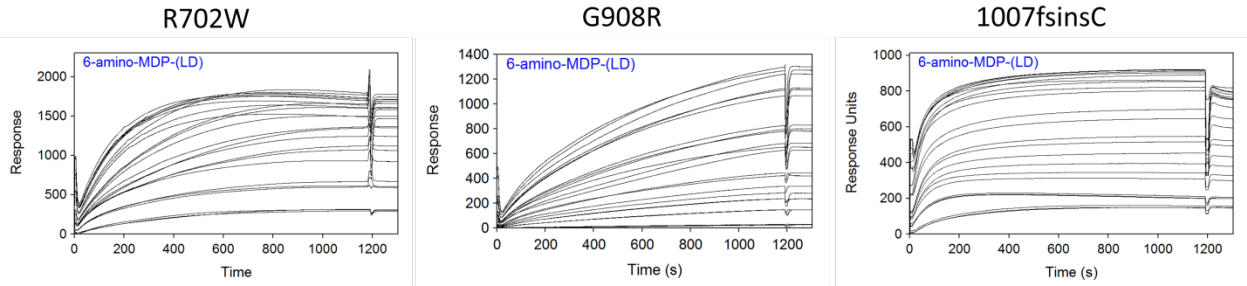


Figure S4. Sensorgrams of WT Nod2 applied to the ligand surfaces of the peptidoglycan library. Sensorgrams processed using Scrubber2 (BioLogic Software).

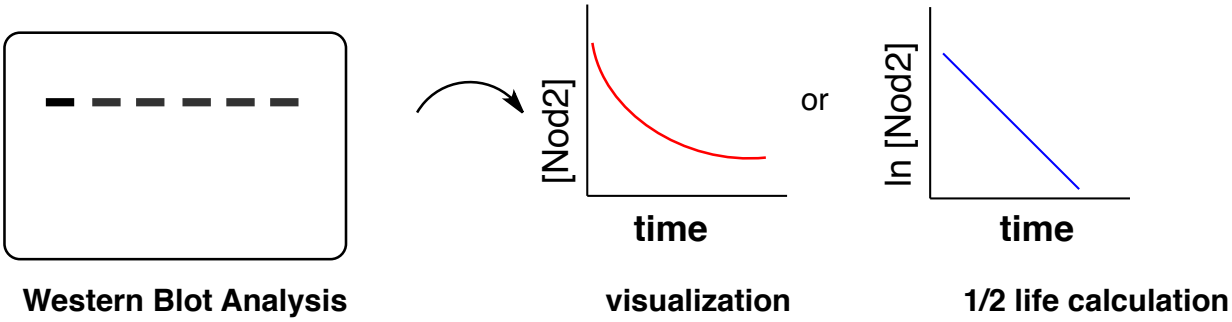
Data analysis – Data was analyzed using Scrubber (BioLogic Software) and binding curves were generated from SimgaPlot (Systate Software). Sensorgrams were double referenced against an untransformed SAM blank lane (on the same chip) and blank injections run in sequence to correct for artifacts and non-specific binding. Nod2-ligand interactions were analyzed by equilibrium analysis and fit to a non-linear model to obtain apparent K_D values.

IV. Limited Proteolysis

Trypsin mediated degradation – Limited proteolysis experiments were performed on a final concentration of 80 nM of purified Nod2 in buffer (50 mM Tris Buffer pH 7.0, 150 mM NaCl and 1 mM EDTA) in the presence or absence of 20 μM cofactors (ATP, ADP, or MDP) and/or 500 nM of Hsp70 construct. Total protein mass in solution was controlled for by the addition of bovine serum albumin (BSA) to account for any effects of excess protein. Samples were treated with trypsin (Promega, sequence grade; 1:200 dilution) and mediated digestion was quenched at set time points (0, 20, 40, 60, 80 min) by adding 3 μL of 5X SDS-Sample buffer (250 mM TrisHCl pH 6.8, 10% SDS, 30% glycerol 0.02% bromophenol blue, 5% BME) to 12 μL of sample. The quenched samples were heated for 10 min at 100 °C and centrifuged. Samples were analyzed by 7.5% SDS-PAGE and Western Blotting, and bands were quantified using Image Lab™. Briefly, samples were electrophoresed in 7.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Western transfer onto PVDF membrane was carried out using semi-dry transfer at 25 V for 1 h. 10% nonfat dry milk in tris-buffered saline-Tween (TBS-T) was used to block the membrane for 1 h and washed in TBS-T 3 times for 5 min each. The blots were incubated overnight at 4°C with the appropriate amount of antibodies prepared in 1% milk. After 3x5 min rinses in TBS-T, the membrane was incubated with HRP-conjugated secondary antibody for 60 min at room temperature. Following secondary incubation, the blot was washed 3 times in TBS-T and incubated with the substrate (GE) according to the manufacturer's instruction. The blots were exposed to Fuji Super RX-U Half Speed Blue films (Brandywine Imaging Inc) in the dark room. Representative blots for each condition are included in Figure S5. The same protocol was used with Nod2 constructs without the addition of trypsin as a control (Table S2).

Data analysis – Data presented are mean ± standard error of the estimate (SE) of at least three independent biological repeats which were analyzed by western blots. Band intensity of Nod2 was quantified and the relative band intensity for each condition was plotted (Figure S6). Each condition was run in triplicate. Nod2 half-life was calculated assuming first-order decay by plotting ($ln[Nod2]$ vs. t) (34, 52). The rate constant was calculated using the slope of the line ($k = -slope$), and the corresponding half-life was determined by $T_{1/2} = \ln(2)/k$. The standard error was calculated using linear regression analysis.

Scheme S1: Data flow for Limited Proteolysis Experiments: Relative Nod2 concentrations were determined from western blots. Normalized Nod2 concentrations were either graphed as [Nod2] vs time or ln [Nod2] vs time. The first graph is useful to visualize decay and the second is used to calculated the ½ life values reported in Tables 1 and 2.



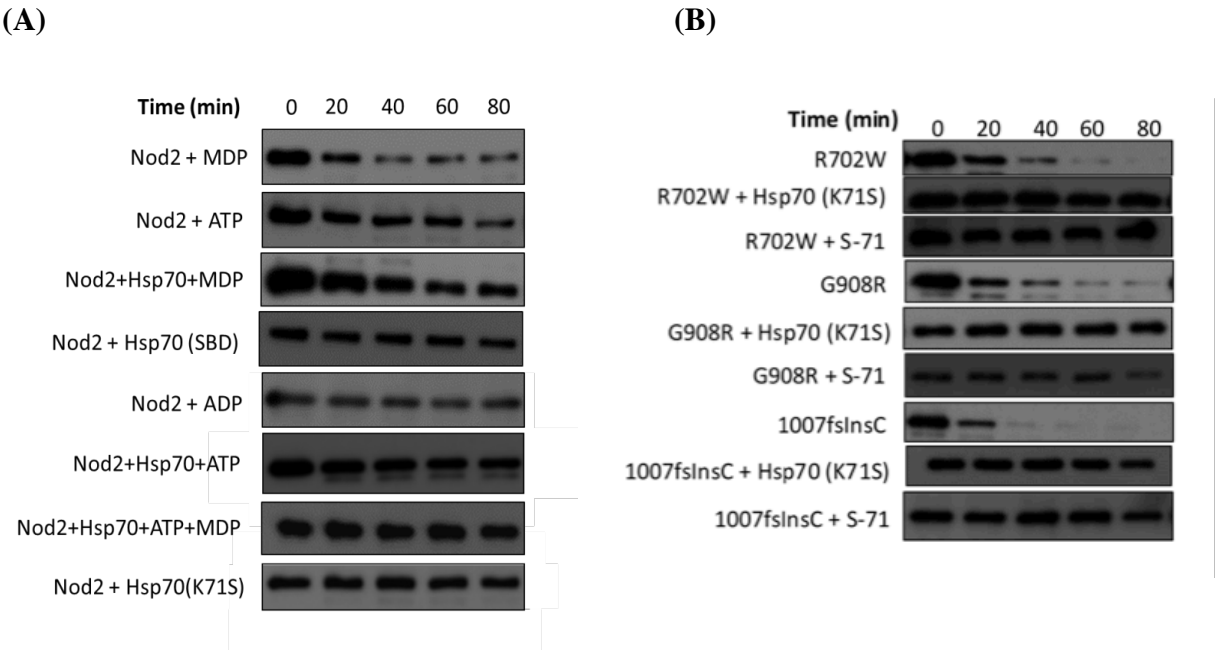


Figure S5. Western blots of trypsin mediated degradation of (A) Nod2 with various cofactors and chaperone proteins and (B) Crohn's associated Nod2 with Hsp70 (K71S) or S-71. Limited proteolysis experiment was performed and Nod2 was probed using a Western blot. Total molecular weight of each condition was normalized with added BSA. Band intensity of Nod2 was quantified and the relative band intensity for each condition was plotted. Each condition was run in triplicate.

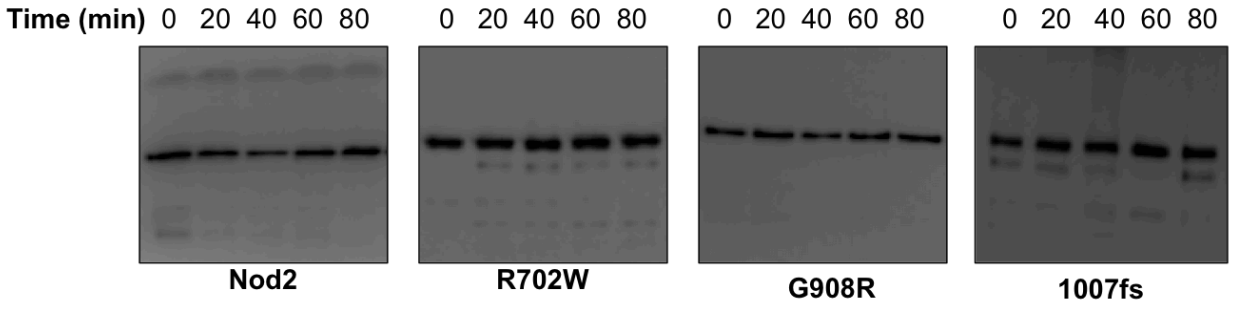


Figure S6. Nod2 and CD associated variants do not degrade in the absence of trypsin: Western blots of Nod2 without the addition of trypsin. Nod2 constructs were subjected to heat for the specific time and run on a gel. Each condition was run in triplicate.

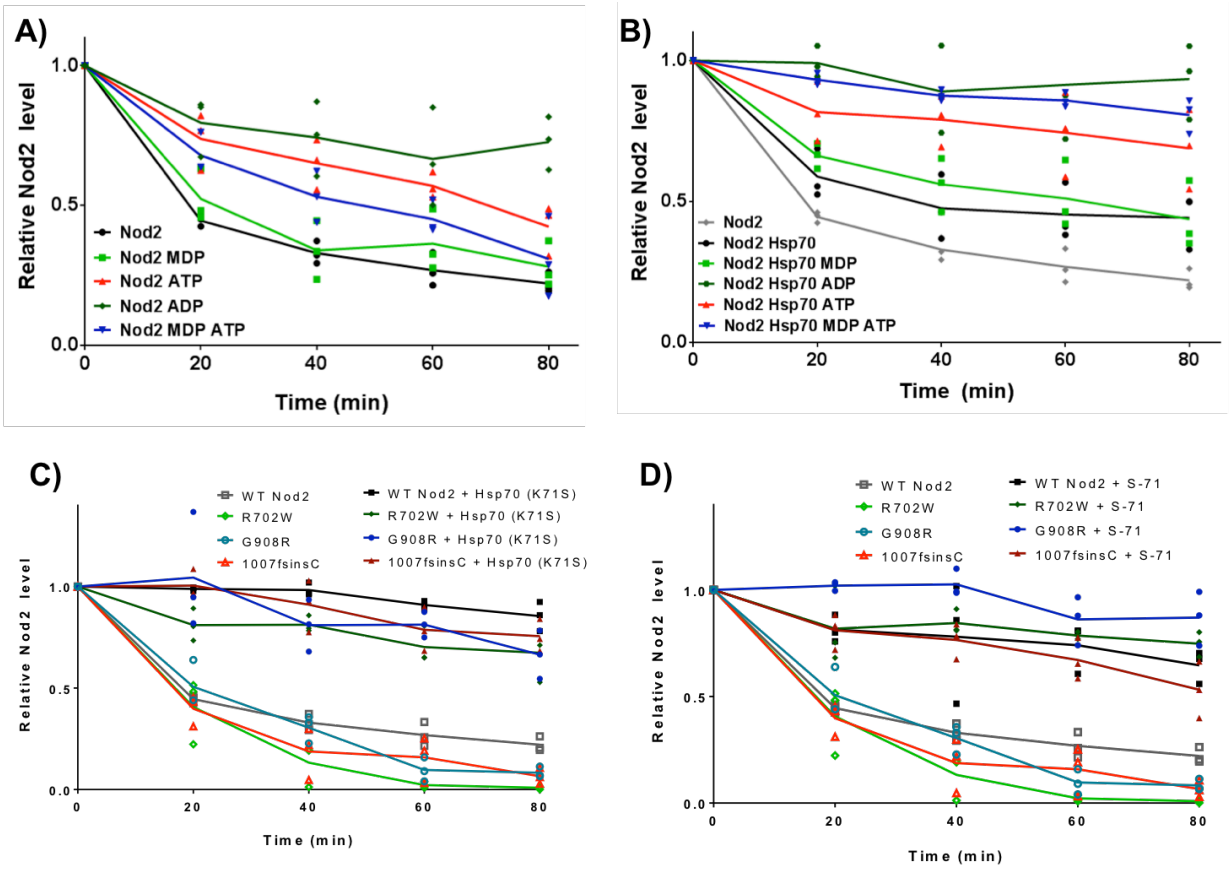


Figure S7(A-D). Relative Nod2 levels over time. Band intensity (relative to band at t = 0) vs. time for each limited proteolysis condition was plotted: (A) WT Nod2 with cofactors, (B) WT Nod2 in the presence of Hsp70 with cofactors, (C) WT and mutant Nod2 in the presence of Hsp70 K71S, (D) WT and mutant Nod2 in the presence of S71. Each replicate is show as an individual point; the connecting lines were added for ease of visualization.

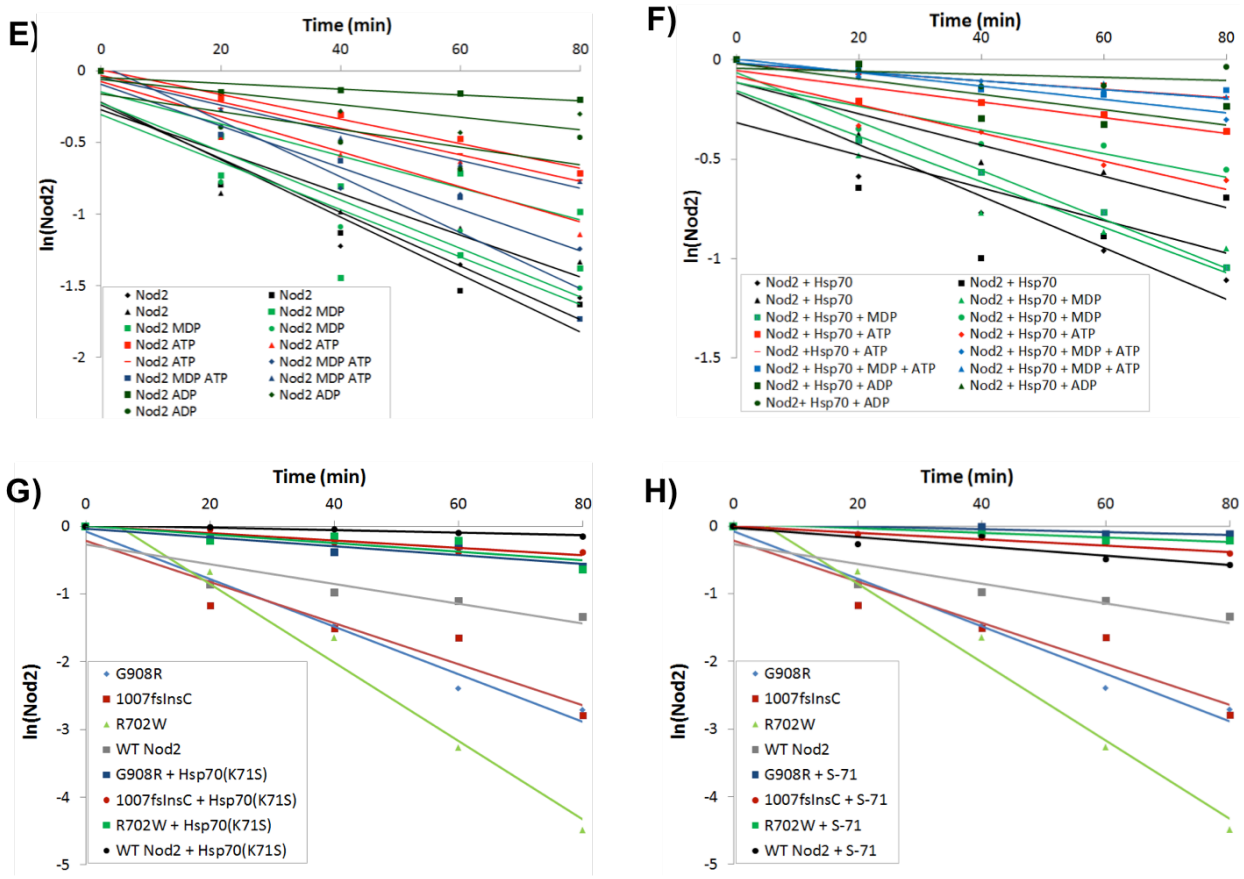


Figure S7 (E-H). Half-life determination: LN [Nod2] vs time graphs for: E) WT Nod2 with cofactors, (F) WT Nod2 in the presence of Hsp70 with cofactors, (G) WT and mutant Nod2 in the presence of Hsp70 K71S, (H) WT and mutant Nod2 in the presence of S71. Plots and best fit lines of each replicate are included on the graph, E and H only show one representative plot and best fit line for each condition. The data were analyzed as described in the "Data analysis" section assuming first order decay rates

V. NF-κB luciferase reporter assay

HEK293T cells were cultured in DMEM, 10% FBS (Atlantic Biologicals), 2 mM glutamine, 1% penicillin/streptomycin and grown in a humidified incubator at 37°C and 5% CO_2. Cells were transfected for 20 hours with Lipofectamine LTX reagent (Invitrogen), 10 ng/well pGL4.10 NF-κB luciferase reporter plasmid, 1 ng/well *Renilla* luciferase plasmind, 2 ng/well Nod2 construct (R702W, G908R, or 1007fs), and 50 ng/well Hsp70 construct (Hsp70, K71S, SBD, or S-71). Activity was induced by the addition of 20 μM MDP-(LD) or blank (water); cells were treated for eight hours after which relative NF-κB luciferase activity was measured using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions.

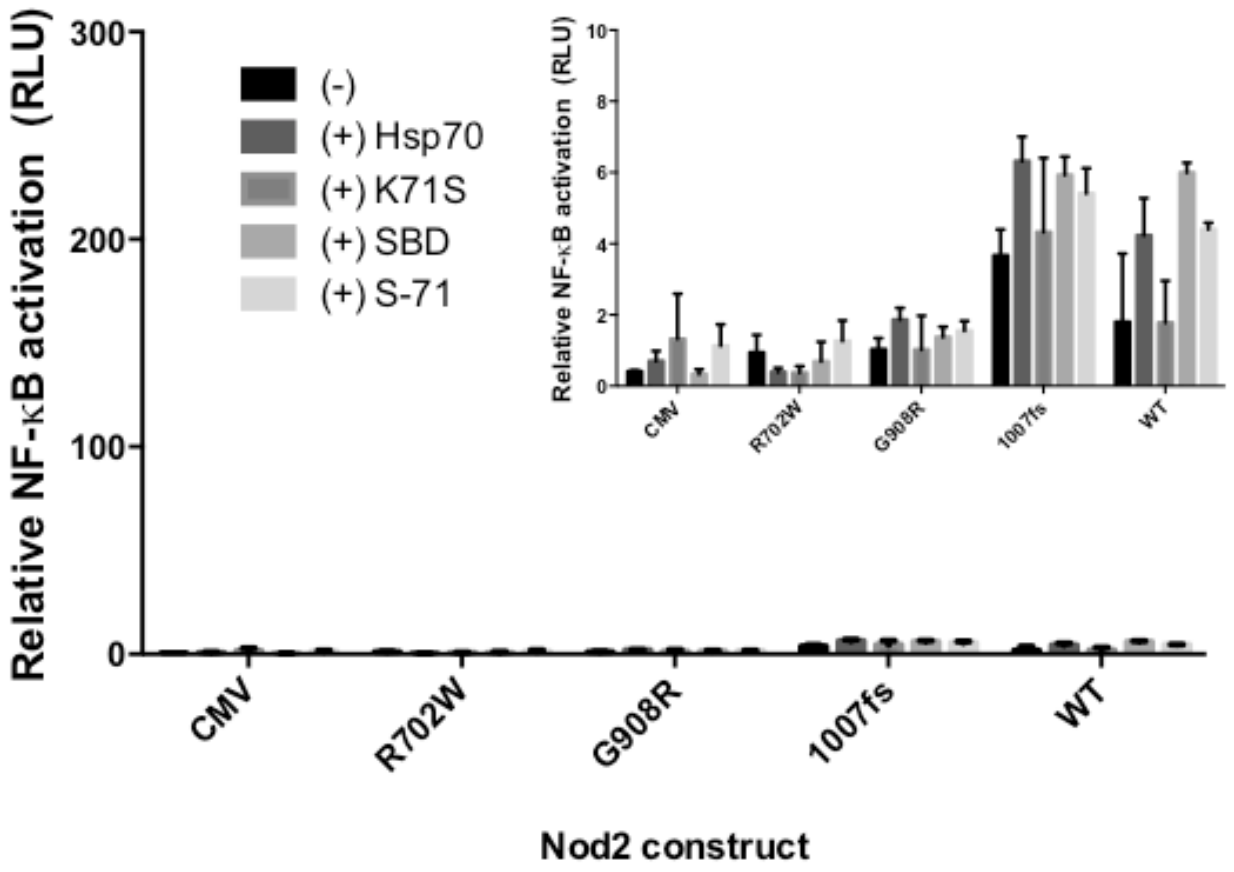


Figure S8: NF-ϰB activation of Nod2 variants (in the absence of MDP) with no chaperones or in the presence (+) of Hsp70 constructs.

VI. Circular Dichroism

CD spectra were taken in a JASCO J715 spectropolarimeter with the protein solution contained in 0.1 cm path length cylindrical cell. Spectra were collected at 1.0 nm intervals over the wavelength range from 260 to 200 nm. Measurements were made to 200 nm (detection limit). Four scans were averaged. The samples were analyzed under native conditions (10 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl). The protein samples were allowed to equilibrate to room temperature before being analyzed at 25 °C in the instrument.

Molar ellipticity ([θ]) was calculated using [θ] = θ/nCl where n is the number of residues in the protein, C is the molar concentration (dM) and l is the pathlength (cm). The ellipticity of the sample was corrected using the ellipticity of the buffer (10 mM sodium phosphate buffer, pH 7.4, containing 50 mM NaCl).

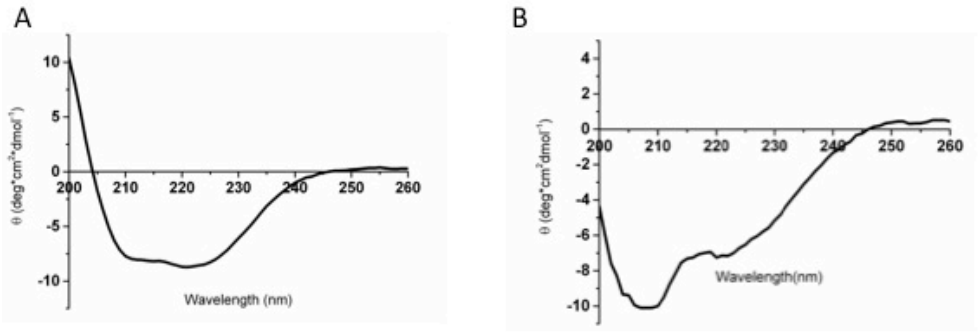


Figure S9. The CD spectrum of the Hsp70 domain constructs: CD spectra of (A) Substrate Binding Domain, and (B) subunit S-71 of the substrate binding domain. Spectra correlate to well-folded proteins.

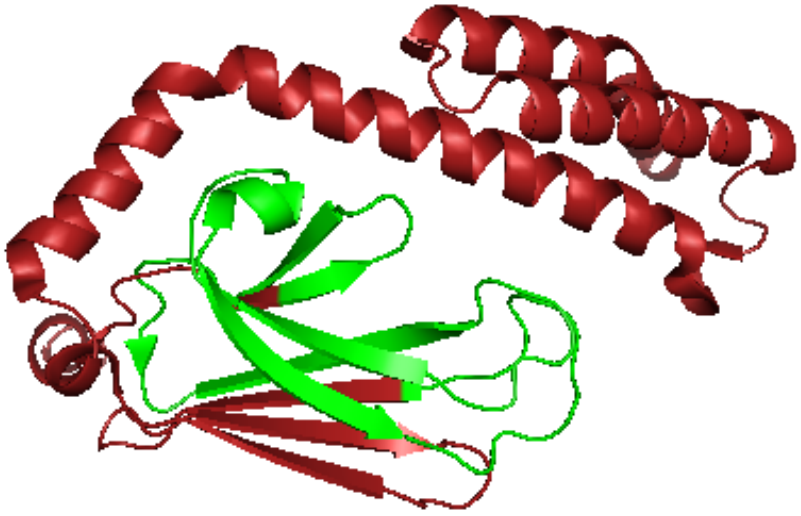


Figure S10. S-71 region (403 AA – 474 AA) of the substrate binding domain that was selected is highlighted in green on the above structure (PDB: 4PO2).

VII. Mass Spectrometry of Select Proteins:

LC-MS/MS analysis of selected proteins were obtained using a Waters Xevo G2-S QTof equipped with a Waters UPLC column. Samples were concentrated to 20 μM prior to application to the LC-MS.

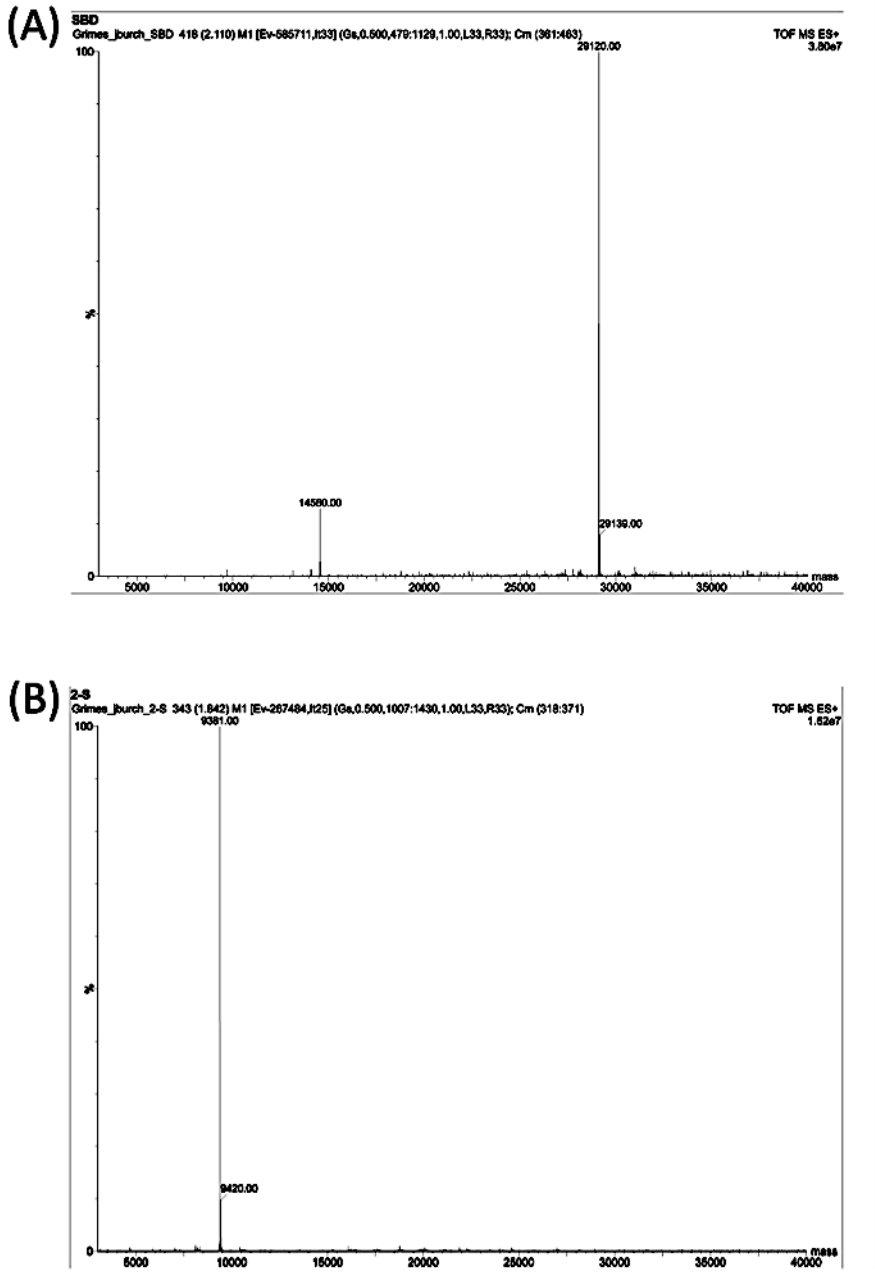


Figure S11: Mass of HSP70 truncations correlation to predicted molecular weights. Mass spectra of (A) Substrate Binding Domain (predicted: 28,707 D found: 29,120 D , and (B) subunit S-71 of the substrate binding domain (predicted: 9,381 D found: 9,381 D).

VIII. References

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