

Supplementary Materials

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Appendix I: ADA2 expression and activity in a patient with PMM2-CDG

Appendix II: Pathogenic ADA2 protein variants form intracellular dimers

References (37–42)

The following Supplementary Material is provided as a separate file:

Supplementary Table S1

Supplementary Materials and Methods

Sanger sequencing

Genomic DNA samples were prepared from heparinized peripheral blood or 1×10^6 Jurkat cells following the instructions of the QIAamp DNA Blood Mini kit (#51104; QIAGEN). Primers were designed with the help of Oligo Primer Analysis Software version 7 (Molecular Biology Insights). ADA2-specific gDNA amplification was performed using Platinum™ SuperFi™ PCR Master Mix (#12358010; Thermo Fisher Scientific). PCR products were purified using the QIAquick PCR purification kit (#28106; QIAGEN). Sanger sequencing was performed on an ABI 3730 XL Genetic Analyzer (Applied Biosystems) at LGC Genomics (Berlin, Germany). Sequencing data were analysed using Chromas 2.6.5 (<http://www.technelysium.com.au>).

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Lymphoprep™ (#1114546; PROGEN) in SepMate™ isolation tubes (#85450; STEMCELL Technologies) according to the manufacturer's instructions. Prior to monocyte isolation, the final centrifugation step was performed at 300xg for 10 minutes at 4°C. CD14+ monocytes were isolated magnetically by positive selection using human CD14 MicroBeads (#130-050-201; Miltenyi Biotec) on LS columns (#130-042-401; Miltenyi Biotec) according to the manufacturer's protocol. The cells were eluted into complete medium (RPMI 1640 medium (#61870044; Gibco) supplemented with 10% fetal calf serum (FCS) (#S181BH-500, Biowest) and 1% penicillin-streptomycin (#15140122; Gibco). Purity > 97% of CD14+ monocytes after magnetic sorting was verified by flow cytometry (anti-CD14-FITC clone M ϕ P9, 1:40, #345784; BD Biosciences). For macrophage differentiation, 3×10^5 CD14+ monocytes were seeded in a 12-well plate in 1 mL complete medium containing 20 ng/mL GM-CSF (#300-03; Peprotech) or 50 ng/mL M-CSF (#300-25; Peprotech). The cells were differentiated for ten days, with medium changes every three days. U-937, THP-1 and Jurkat cells (all purchased from ATCC) were cultured in complete RPMI medium. HEK293T cells (ATCC) were cultured in DMEM supplemented with 10% FCS and 1% penicillin-streptomycin. For transfection, the cells were seeded in medium containing no antibiotics.

Inhibition of the secretory pathway was achieved by 24-hour incubation with 1 μ g/mL brefeldin A (#00-4506-51; Thermo Fisher Scientific) or 2 μ g/mL monensin (GolgiStop™, #554724, BD Biosciences). For analysis of N-glycosylation and glycan trimming, the cells were incubated with tunicamycin (#T7765; Sigma-Aldrich) at 2.5 μ g/mL (HEK293T cells) or 5 μ g/mL (HMDM) and castanospermine (#BML-S107-0100; Enzo Life Sciences) at 100 μ g/mL for 24h or 48h. α -mannosidases were inhibited by 72-96h incubation with 375 nM kifunensine (#K1140; Sigma-

Aldrich) and/or 10 μ M swainsonine (#S8195; Sigma-Aldrich). Monocytes were treated with 1 μ g/mL tunicamycin for 24h for ER stress induction. Cells were treated with 50 μ M chloroquine (#C6628; Sigma-Aldrich) for 24h to inhibit autophagy. Proteasome inhibition was achieved by 14 to 24-hour incubation with 200 nM delanzomib (#S1157; Selleck Chemicals GmbH). HSP90 was inhibited by 24-hour incubation with 360 nM geldanamycin (#G3381; Sigma-Aldrich).

Microscopy

HMDM were differentiated with GM-CSF or M-CSF for 10 days in a 12-well plate as described above. Cell morphology was evaluated in the culture plate by brightfield microscopy on an EVOS M7000 Microscope Imaging System (#AMF7000; Invitrogen) using the 20X objective.

Transfection

The plasmid expressing myc-DDK-tagged wild-type ADA2 (transcript variant 3, NM_001282225) was purchased from OriGene Technologies (#RC238645). Pathogenic ADA2 variants were created by site-directed mutagenesis using the Q5® Site-Directed Mutagenesis Kit (#E0554; New England Biolabs) according to the manufacturer's instructions. Stable competent *E. coli* (#C3040H, New England Biolabs) were transformed with the generated constructs. Plasmid DNA was purified with the help of the QIAprep Spin Miniprep Kit (#27104, QIAGEN). Successful mutagenesis was verified by Sanger sequencing (LGC Genomics). HEK293T cells were seeded at 5×10^4 cells/well in 1 mL in a 24-well plate or 2.5×10^5 cells/well in 2 mL in a 6-well plate 24h prior to transfection. The cells were transfected with 5 ng and 25 ng plasmid DNA, respectively, using Lipofectamine™ 2000 Transfection Reagent (#11668019; Invitrogen) according to the manufacturer's instructions. The medium was changed after 24h. Cells and supernatants were collected 48h after transfection.

Generation of ADA2^{-/-} cell lines by CRISPR/Cas9

Single-guide RNAs targeting ADA2 from the human CRISPR Brunello library (#73179; addgene) (37) were cloned into the lentiCRISPRv2 puro plasmid. lentiCRISPRv2 puro was a gift from Brett Stringer (plasmid #98290; addgene; <http://n2t.net/addgene:98290>; RRID: Addgene_98290). Jurkat cells were transfected by electroporation using the Neon™ Transfection System (#MPK5000; Thermo Fisher Scientific) according to the manufacturer's instructions: 2×10^5 cells were resuspended in Resuspension Buffer R and mixed with 1 μ g plasmid DNA. Electroporation was achieved with the following conditions: 1325V, 10ms, 3 pulses. After electroporation, the cells

were directly transferred into 500 μ L prewarmed complete RPMI 1640 medium. On day 1 after transfection, puromycin (#ant-pr-1; InvivoGen) was added at a final concentration of 1 μ g/mL. After 36h, cells were seeded at 1 cell/well in 100 μ L RPMI 1640 medium supplemented with 20% FCS in a round bottom 96-well plate. After 14 days, the clones were screened for knock out of ADA2 by western blot.

Transduction

Pathogenic ADA2 variants were created by site-directed mutagenesis as described above. Fragments containing the sequence coding for the untagged ADA2 protein were then generated using the following primers and CloneAmp™ HiFi PCR Premix (#639298; Takara Bio):

ORF_ADA2_PSPXI_F_GGTGGTACTCGAGTATGTTGGTGGATGGCCCATCTGA;

ORF_ADA2_PmeI_R_GGTGGTGT*TTAAACCT*TTGTAGCCACATCTGCTATGAACT*.

The fragments were cleaned up using the QIAquick PCR Purification Kit (#28104; QIAGEN) and cloned into the p3D lentiviral expression vector as follows: Restriction of the expression vector was performed in CutSmart buffer (#B7204; New England Biolabs) with PspX1 (#R0656; New England Biolabs) and PmeI (#R0560; New England Biolabs) at 37°C overnight followed by 20-minute heat inactivation at 65°C. Dephosphorylation of 5'-ends was performed using rSAP (#M0371; New England Biolabs) at 37°C overnight followed by 20-minute heat inactivation at 65°C. The PCR fragments were subsequently inserted into the expression vector using T4 ligase (#M0202; New England Biolabs) for 10 minutes room temperature followed by 10-minute heat inactivation at 65°C. After DNA cleanup (#28104; QIAGEN), stable competent *E. coli* (#C3040H, New England Biolabs) were transformed with the generated plasmids. Plasmid DNA was purified with the help of the QIAprep Spin Miniprep Kit (#27104, QIAGEN). Sanger sequencing (LGC Genomics) was performed to confirm that the genetic information for ADA2 was intact and that the respective mutations were present. Lentiviral particles were generated by transfecting HEK293T cells with the plasmids p3A (Gag-Pol), p3B (Rev), p3C (VSV-G) and p3D (expression vector) at a ratio of 1:1:1:3 using Lipofectamine™ 2000 Transfection Reagent (#11668019; Invitrogen). Supernatants containing lentiviral particles were harvested 48h and 72h after transfection. The viral titer was determined using the qPCR Lentivirus Titer Kit (#LV900; Applied Biological Materials). Viral supernatants were prepared at MOI=40 with 8 μ g/mL polybrene and 10^6 U-937 cells were transduced by spinoculation at 700xg for 2 hours at 32°C. After 4 hours at 37°C, the supernatant was replaced by fresh complete RPMI medium. Selection with 1 μ g/mL puromycin (#ant-pr-1; InvivoGen) was started 48h after the transduction.

Subcellular fractionation

Membrane and cytosolic fractions were generated from 10×10^6 U-937 cells using the Mem-PER™ Plus Membrane Protein Extraction Kit (#89842; Thermo Fisher Scientific). Incubation times were optimized to increase protein yield to 45 minutes for the permeabilization buffer and 90 minutes for the solubilization buffer. The lysosomal fraction was produced with the help of the Lysosome Enrichment Kit for Tissues and Cultured Cells (#89839; Thermo Fisher Scientific) according to the manufacturer's protocol.

Antibodies for immunoblotting

The membranes were probed with the following primary antibodies: anti-ADA2 (clone EPR25430-131, #ab288296, 1:1000; abcam), anti-ADA2 (#HPA007888, 1:500; Sigma-Aldrich; RRID: AB_1078495), Clone OTI4C5, anti-DDK (FLAG) (clone OTI4C5, #TA50011, 1:1000; OriGene Technologies; RRID: AB_2622345), anti-LC3B (#ab51520, 1:1000; abcam; RRID: AB_881429), anti-BiP (#3183, 1:1000; Cell Signaling; RRID: AB_10695864), anti-LAMP-1 (clone H4A3, #sc-20011; 1:500; Santa Cruz Biotechnology; RRID: AB_626853), anti-CD98 (clone E-5, #sc-376815, 1:500; Santa Cruz Biotechnology; RRID: AB_2938854), anti-GAPDH (clone 7B, #sc-69778, 1:500; Santa Cruz Biotechnology; RRID: AB_1124759), anti- β -actin (clone: AC-15, #A5441, 1:9,000; Sigma-Aldrich; RRID: AB_476744) at 4°C overnight or at room temperature for two hours. The membranes were washed and incubated with the respective HRP-coupled secondary antibodies for one hour at room temperature: goat anti-rabbit IgG H&L (#ab205718, 1:5000; abcam; RRID: AB_2819160), Goat Anti-Mouse IgG (H + L) (#71045, 1:5000; Sigma-Aldrich; RRID: AB_11211441) or VeriBlot Detection Reagent (#ab131366, 1:1000 in tris saline/5% skim milk; abcam).

The anti-ADA2 antibody (clone EPR25430-131, #ab288296, 1:1000; abcam) was used in the following figures of the article: Fig. 1A-D, Fig. 3C+D, Fig. 4A+B, Fig. 6A+B, Fig. 7A, B(far right panel), C-E, and Fig. A1. The anti-ADA2 antibody (#HPA007888, 1:500; Sigma-Aldrich; RRID: AB_1078495) was applied in the remaining blots: Fig. 2A-D, Fig. 3A+B, Fig. 6C, and Fig. 7B+E.

Immunoprecipitation

Whole cell lysates were prepared from HEK293T cells transfected with wild-type ADA2 or the variants p.R169Q or HMDM from HC or DADA2 patients in NP-40 lysis buffer at a protein concentration of 1 mg/mL. Primary antibodies were added to 1000 μ g lysate or 500 μ g lysate, respectively, and incubated rotating at 4°C overnight. Alternatively, 1 mL supernatant was directly incubated with the primary antibodies. After overexpression, tagged ADA2 was pulled down using

1 µg anti-DDK (FLAG) clone OTI4C5 (#TA50011, OriGene Technologies). An isotype control sample was incubated with 1 µg mouse IgG1 (#02-6100; Invitrogen) in parallel. Endogenous ADA2 was pulled down using 1 µg anti-ADA2 (clone EPR25430-131, #ab288296, abcam) with rabbit monoclonal IgG (clone EPR25A, #ab172730; abcam) used as isotype control. For immunoprecipitation, the samples were rotated with 100 µL SureBeads™ Protein G Magnetic Beads (#1614023, Bio-Rad) for 1h at room temperature. The beads were magnetized and washed four times with PBS/0.1% Tween 20. Samples for mass spectrometric analysis were washed twice with NP-40 lysis buffer and twice with PBS. For western blot, the samples were eluted from the beads in Bolt™ LDS sample buffer (#B0007; Thermo Fisher Scientific).

Protein digest

Beads from immunoprecipitation experiments were resuspended in 20 µL denaturation buffer (6 M Urea, 2 M Thiourea, 10 mM HEPES, pH 8.0), reduced for 30 min at 25°C in 12 mM dithiothreitol, followed by alkylation with 40 mM chloroacetamide for 20 min at 25°C. Samples were first digested with 0.5 µg endopeptidase LysC (Wako, Osaka, Japan) for 4h. After diluting the samples with 80 µL 50 mM ammonium bicarbonate (pH 8.5), 1 µg sequence grade trypsin (Promega) was added overnight at 25°C. The peptide-containing supernatant was collected and acidified with formic acid (1% final concentration) to stop the digestion. Peptides were desalted and cleaned up using Stage Tip protocol (38). After elution with 80% acetonitrile/0.1% formic acid, samples were dried using speedvac, resolved in 3% acetonitrile/0.1% formic acid and analysed by LC-MS/MS.

LC-MS/MS analyses

Peptides were separated on a reversed-phase column (20 cm fritless silica microcolumns with an inner diameter of 75 µm, packed with ReproSil-Pur C18-AQ 3 µm resin (Dr. Maisch GmbH) using a 90 min gradient with a 250 nL/min flow rate of increasing Buffer B concentration (from 2% to 60%) on a High-Performance Liquid Chromatography (HPLC) system (Thermo Fisher Scientific) and ionized using an electrospray ionization (ESI) source (Thermo Fisher Scientific) and analyzed on a Thermo Q Exactive HF-X instrument (*HEK293T*) or on a Q Exactive Plus instrument (*HMDM*). The Q Exactive HF-X instrument was run in data dependent mode selecting the top 20 most intense ions in the MS full scans, selecting ions from 350 to 2000 m/z, using 60 K resolution with a 3×10^6 ion count target and 10 ms injection time. Tandem MS was performed at a resolution of 15 K. The MS2 ion count target was set to 1×10^5 with a maximum injection time of 100 ms. Only precursors with charge state 2–6 were selected for MS2. The dynamic exclusion duration was

set to 30 s with a 10-ppm tolerance around the selected precursor and its isotopes. The Q Exactive Plus instrument was run in data dependent mode selecting the top 10 most intense ions in the MS full scans, selecting ions from 350 to 2000 m/z, using 70 K resolution with a 3×10^6 ion count target and 50 ms injection time. Tandem MS was performed at a resolution of 17.5 K. The MS2 ion count target was set to 5×10^4 with a maximum injection time of 250 ms. Only precursors with charge state 2–6 were selected for MS2. The dynamic exclusion duration was set to 30 s with a 10-ppm tolerance around the selected precursor and its isotopes.

Raw data were analyzed using MaxQuant software package (v1.6.3.4) (39). The internal Andromeda search engine was used to search MS2 spectra against a human UniProt database (HUMAN.2020-06), including ADA2 mutant sequence, containing forward and reverse sequences. The search included variable modifications of methionine oxidation, N-terminal acetylation and fixed modification of carbamidomethyl cysteine. The FDR was set to 1% for peptide and protein identifications. Unique and razor peptides were considered for quantification. The LFQ (label-free quantitation) algorithm was activated. The resulting text file was filtered to exclude reverse database hits, potential contaminants, and proteins only identified by site.

SomaScan Assay

Serum samples (55 μ L) from 12 healthy controls, 9 heterozygous ADA2 carriers and 12 DADA2 patients were analyzed by the 7,000-plex SomaScan[®] Assay (SomaLogic, Inc.). One carrier was identified as an outlier by principal component analysis and excluded from further analysis. For two carriers and 10 patients, samples from multiple time points were included. The cohort details are presented in Table S2. The assay readout was provided in relative fluorescent units (RFU) for all measured aptamers for each serum sample.

ADA2 enzyme activity

Adenosine deaminase 2 enzyme activity was determined in whole cell lysates from HEK293T cells overexpressing ADA2 or HMDM with and without glycan removal as well as in supernatants from transfected HEK293T cells and human serum. Deaminase activity was measured in a colorimetric assay adapted from Giusti and Galanti (40). Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (#E114; Sigma-Aldrich) was used to inhibit ADA1 activity. Triplicate measurements were performed for all samples. Enzymatic activity of pathogenic ADA2 variants overexpressed HEK293T cells was normalized to the activity of wild-type ADA2.

qPCR

5-10x10⁵ cells were lysed in TRIzol™ Reagent (#15596018; Thermo Fisher Scientific) for 3 minutes at room temperature and homogenized before storage at -80°C. To measure the ER stress response, HEK293T cells were seeded at 5x10⁴ cells/ml in a 24-well plate 24h prior to transfection. TRIzol™ samples were prepared 48h after transfection. RNA was extracted using the PureLink™ RNA Mini Kit (#12183018A; Thermo Fisher Scientific) according to the manufacturer's instruction. cDNA was generated from 20 ng RNA using the SuperScript™ VILO™ cDNA Synthesis Kit (#11754050; Thermo Fisher Scientific). Quantitative polymerase chain reaction (qPCR) analysis was performed with SsoAdvanced™ Universal SYBR® Green Supermix (#1725271; Bio-Rad Laboratories) and the following primers:

ADA2(929-1038)_F_GGCTCCGAATCAAGTTCCCC;

ADA2(929-1038)_R_TAAGGCAGCTTAACGCCATCC;

ADA2(1138-1257)_F_ACCAGAATCGGCCATGGA;

ADA2(1138-1257)_R_CTACAGGGTGGTTCCTCAAGTCA;

HSPA5_F_CAAGCAACCAAAGACGCTGGA; HSPA5_R_GCCACCCAGGTCAAACACCA;

DDIT3_F_AGAGGAAGAATCAAAAATCT; DDIT3_R_AGCTCTGACTGGAATCTGGA;

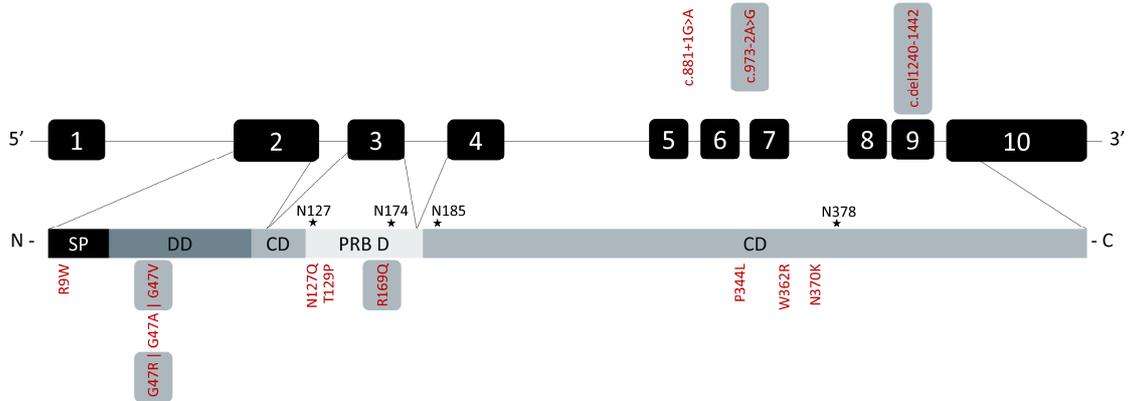
GAPDH_F_GTCTCCTCTGACTTCAACAGCG;

GAPDH_R_ACCACCCTGTTGCTGTAGCCAA. For all conditions, three technical replicates

were measured. The experiment was run on a QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific) and analyzed using the QuantStudio™ Design & Analysis Software v1.5.2. The relative abundance of *ADA2* was normalized to the expression level of *GAPDH*. and different conditions were compared using the 2^{-ΔΔCt} method (41).

Supplementary Figures

A



B

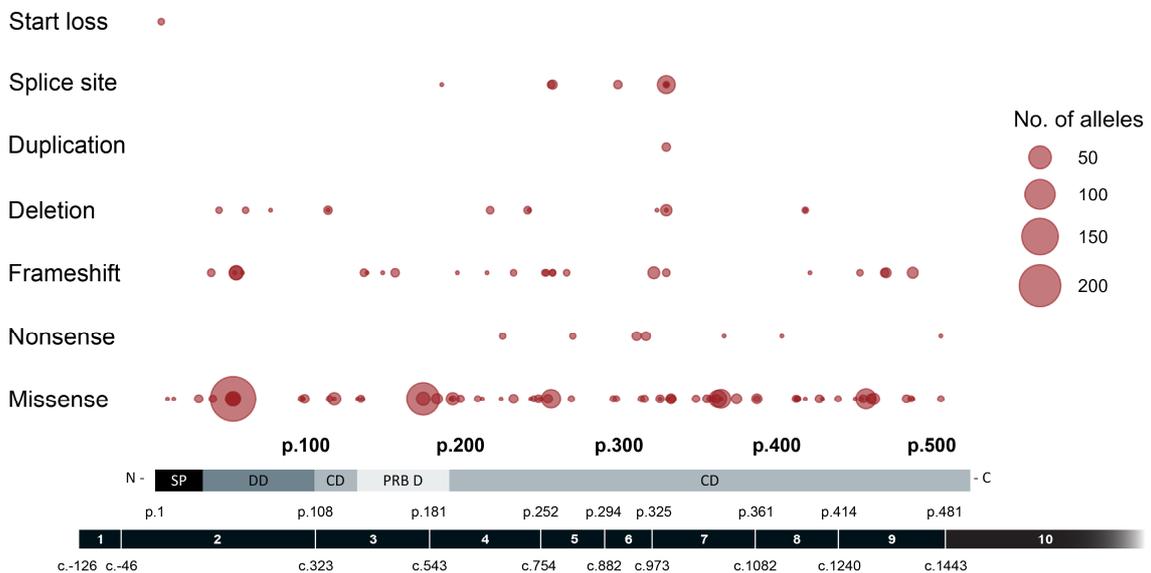


Fig. S1: Pathogenic variants in *ADA2* underlying *ADA2* deficiency. (A) Overview of the location of all pathogenic variants of *ADA2* included in this study across the *ADA2* gene and encoded protein domains. Shaded variants are present in our cohort of DADA2 patients. Stars indicate N-glycosylation sites. (B) Overview of frequency, location and mutation type of all published pathogenic *ADA2* variants as reported by Dzhush and colleagues (7). Legend: CD, catalytic domain; DD, dimerization domain; EV, empty vector; PRB D, putative receptor binding domain.

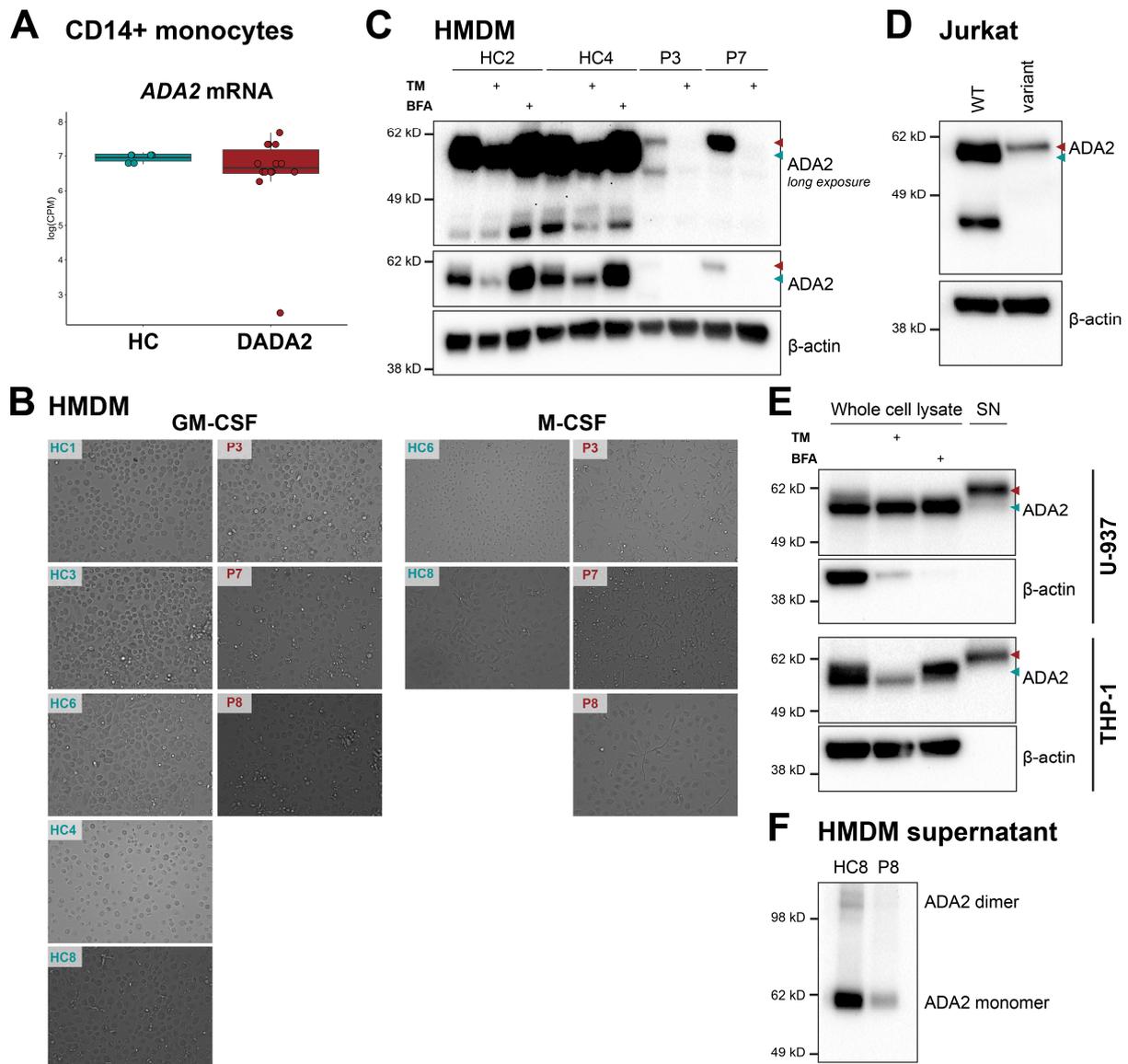


Fig. S2: Expression of ADA2 in primary immune cells and cell lines. (A) *ADA2* mRNA levels in sorted CD14+ monocytes. Single-cell RNA sequencing was performed on magnetically sorted CD14+ monocytes from healthy controls (HC) and DADA2 patients (P). The graph was generated based on the data set published by Watanabe and colleagues (32). Boxplots show median and interquartile range, whiskers extend from the hinge to the highest and lowest value within 1.5 * interquartile range of the hinge. (B) Morphology of DADA2 human monocyte-derived macrophages. CD14+ monocytes from healthy controls (HC) and DADA2 patients (P) were differentiated over ten days with 20 ng/mL GM-CSF (left) or 50 ng/mL M-CSF (right) and imaged by brightfield microscopy on day 10. (C) ADA2 expression in DADA2 human monocyte-derived macrophages. ADA2 protein expression by western blot of whole cell lysates from GM-CSF-differentiated HMDM from healthy controls (HC) and DADA2 patients (P) untreated or treated with 5 μ g/mL tunicamycin (TM) or 1 μ g/mL brefeldin A (BFA) for 24h. (D) ADA2 variant in Jurkat cells. Western blot of whole cell lysates from Jurkat cells expressing wild type (WT) or mutant. ADA2. (E) ADA2 protein expression in whole cell lysates and supernatant (SN) of U-937 and THP-1 cells left untreated or treated with 1 μ g/mL tunicamycin (TM) or 1 μ g/mL brefeldin A (BFA) for 24h. (F) ADA2 secreted from M-CSF-differentiated human monocyte-derived

macrophages from healthy control HC8 and DADA2 patient P8. ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) in all depicted blots.

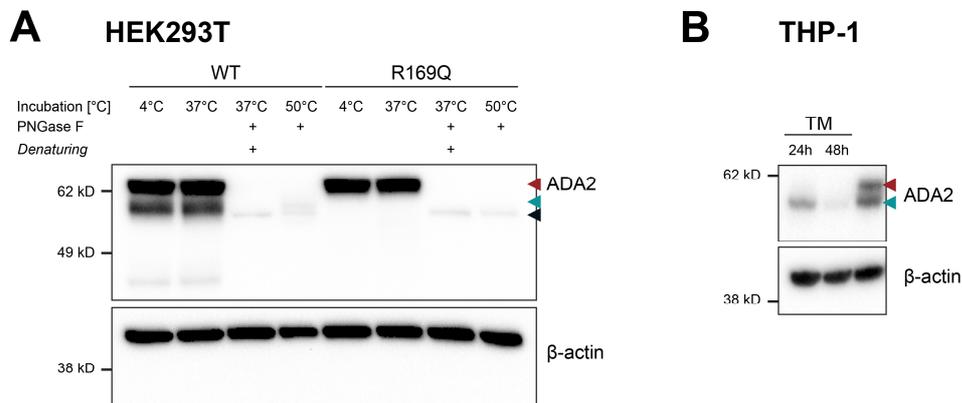


Fig. S3: ADA2 expression upon modification of N-glycosylation (A) Detection of glycan-free ADA2 by the anti-ADA2 antibody clone EPR25430-131. HEK293T cells were transfected with wild-type (WT) ADA2 or the pathogenic variant R169Q. Glycan removal of whole cell lysates was performed under denaturing conditions (#P0704; New England Biolabs) or under non-denaturing conditions (#A39245; Gibco) by incubation with PNGase F for 1 hour at 37°C and 50°C, respectively. (B) THP-1 cells were left untreated or incubated with 2.5 µg/mL tunicamycin for 24h and 0.1 µg/mL tunicamycin for 48h, respectively. The anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) was used for detection in all depicted western blots.

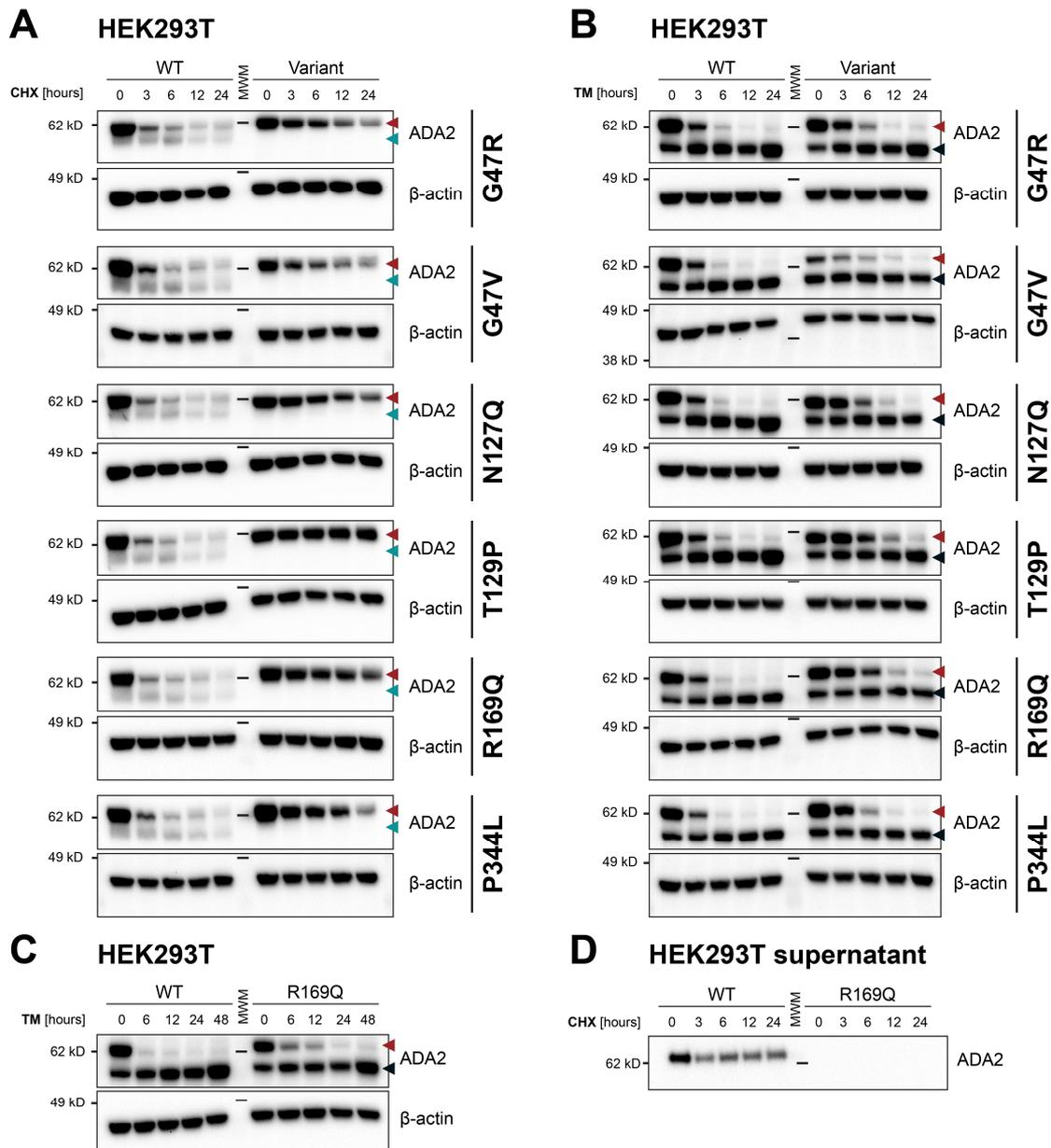


Fig. S4: Stability of LMW-ADA2. (A) HEK293T cells were transfected with wild-type (WT) ADA2 or different pathogenic variants and treated with 500 $\mu\text{g}/\text{mL}$ cycloheximide (CHX) over 24h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. (B) HEK293T cells were transfected with wild-type (WT) ADA2 or different pathogenic variants and treated with 2.5 $\mu\text{g}/\text{mL}$ tunicamycin (TM) over 24h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. (C) HEK293T cells were transfected with wild-type (WT) ADA2 or the pathogenic variant p.R169Q and treated with 2.5 $\mu\text{g}/\text{mL}$ tunicamycin (TM) over 48h. Whole cell lysates were generated at the indicated time points and immunoblotted for ADA2 expression. (D) HEK293T cells were transfected with wild-type (WT) ADA2 or the pathogenic variant p.R169Q and treated with 500 $\mu\text{g}/\text{mL}$ cycloheximide (CHX) over 24h. Supernatant was collected at the indicated time points and ADA2 levels were determined by western blot. Triangles indicate HMW-ADA2 (red), LMW-ADA2 (green) and glycan-free ADA2 (dark blue). In figures A+D, ADA2 was detected using anti-

ADA2 antibody clone EPR25430-131 (#ab288296; abcam); in figures B+C anti-ADA2 (#HPA007888, Sigma-Aldrich) was used. Legend: MWM, molecular weight marker.

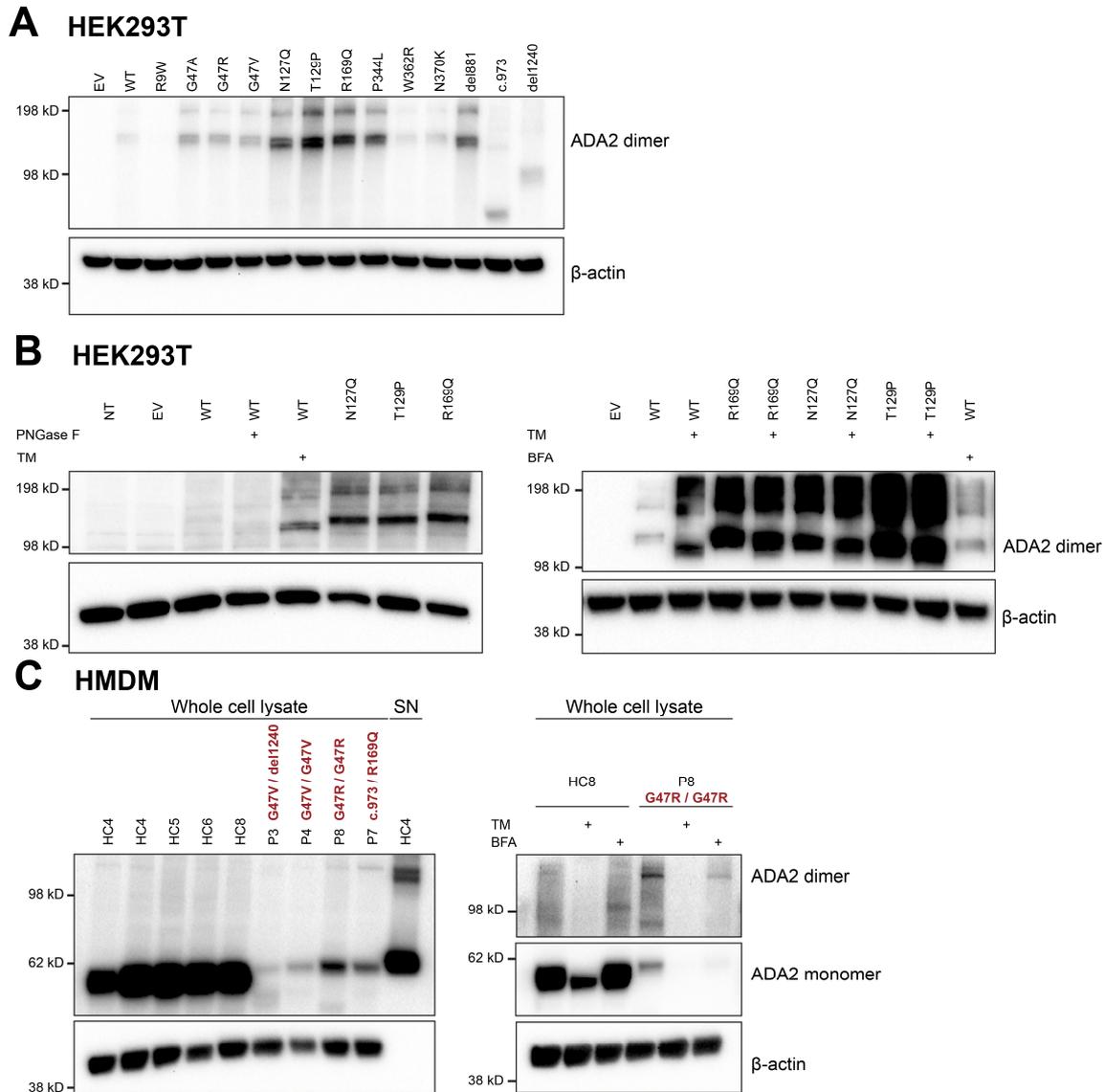


Fig. S5: Intracellular dimer formation mutant ADA2. (A) Formation of intracellular ADA2 dimers in HEK293T cells transfected with wild-type (WT) ADA2 and different pathogenic ADA2 variants. The blot is equivalent to the one shown in Fig. 1D. (B) Formation of intracellular ADA2 dimers in HEK293T cells transfected with wild-type (WT) ADA2 and different pathogenic ADA2 variants with or without treatment with 2.5 $\mu\text{g}/\text{mL}$ tunicamycin (TM) or 1 $\mu\text{g}/\text{mL}$ brefeldin A (BFA). ADA2 was detected using anti-ADA2 (#HPA007888, Sigma-Aldrich) (*left*) or anti-DDK (FLAG) clone OTI4C5 (#TA50011, OriGene Technologies) (*right*). (C) ADA2 protein expression and intracellular dimer formation in GM-CSF (*left*) or M-CSF (*right*) differentiated human monocyte-derived macrophages from healthy controls (HC) and DADA2 patients (P) with or without treatment with 5 $\mu\text{g}/\text{mL}$ tunicamycin (TM) or 1 $\mu\text{g}/\text{mL}$ brefeldin A (BFA) for 24h. In figures A+C, ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam). Legend: NT, non-transfected; EV, empty vector.

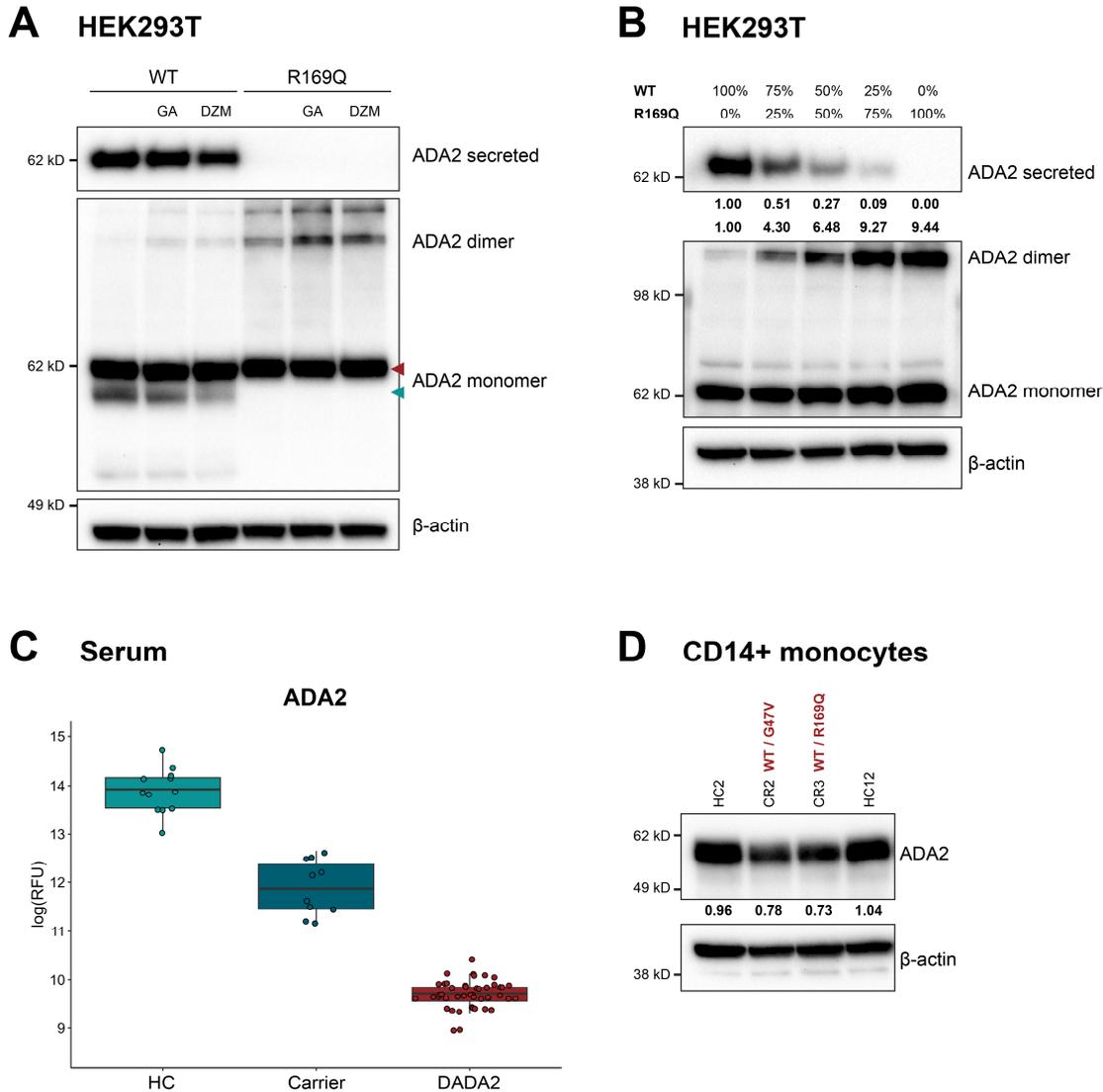
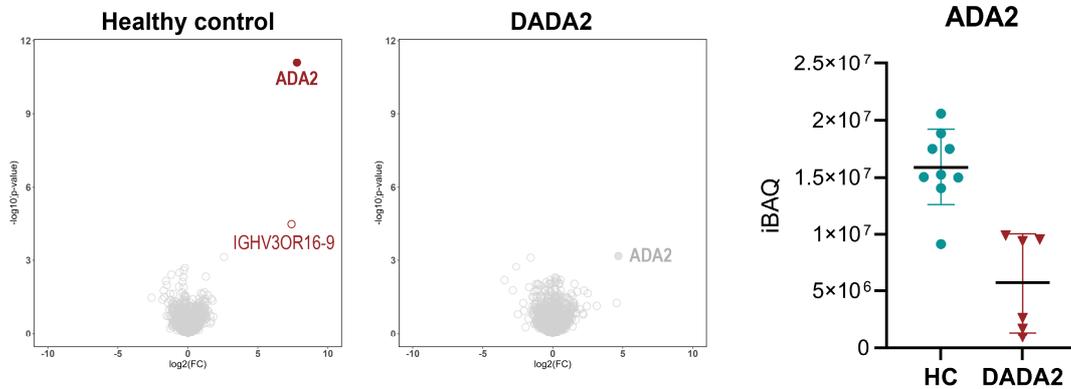


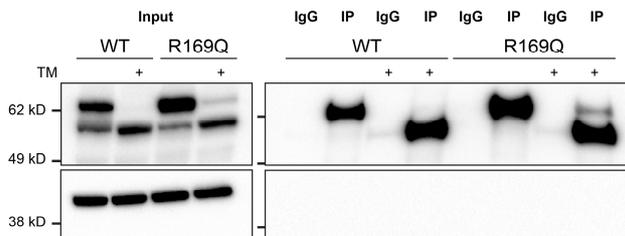
Fig. S6: Aggregate formation impairing secretion of mutant ADA2. (A) ADA2 secretion and formation of intracellular ADA2 dimers in HEK293T cells transfected with wild-type (WT) ADA2 and the pathogenic variant p.R169Q. The cells were left untreated or treated with 360 nM geldanamycin (GA) for 24h or 200 nM delanzomib for 14h. ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam). Triangles indicate HMW-ADA2 (red) and LMW-ADA2 (green). (B) HEK293T cells were transfected with a total of 100 ng plasmid DNA of WT ADA2 or the pathogenic variant p.R169Q at the indicated ratios. ADA2 protein levels were determined in supernatant (*upper blot*) or whole cell lysate (*middle blot*) using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) and anti-DDK (FLAG) clone OTI4C5 (#TA50011, OriGene Technologies), respectively. (C) ADA2 serum protein levels in serum of healthy controls, carriers and DADA2 patient were determined by SomaScan assay. Measurements are depicted as relative fluorescent units (RFU) that are directly proportional to the amount of target protein in the sample. For several patients, multiple samples from different time points were included. Boxplots show median and interquartile range, whiskers extend from the hinge to the highest and lowest value within 1.5 * interquartile range of the hinge. (D) ADA2 protein expression in whole cell lysates of CD14+ monocytes from healthy control (HC) and DADA2 heterozygous carriers

(CR) by western blot, normalized to β -actin. ADA2 was detected using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam).

A HMDM



B HEK293T



C HMDM

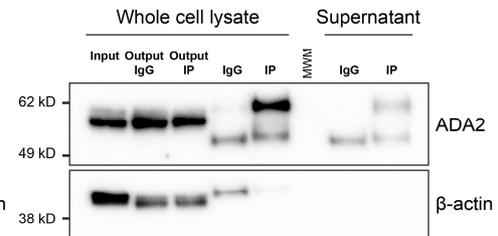


Fig. S7: Immunoprecipitation of ADA2. (A) CD14⁺ monocytes from three healthy controls (HC) and two DADA2 patients (P) were differentiated into macrophages using 20 ng/mL GM-CSF for 10 days. Immunoprecipitation with the anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) was followed by mass spectrometry. Binders significantly pulled down compared to isotype control with a false discovery rate of 10% are highlighted in red. The graph shows abundance of ADA2 in the IP samples of HC HMDM compared to DADA2 HMDM. The dot plots show median and interquartile range. (B) HEK293T cells transfected with wild-type (WT) ADA2 and the pathogenic variant p.R169Q were treated with or without 2.5 μ g/mL tunicamycin (TM) for 24h. ADA2 was pulled down from whole cell lysates using anti-DDK (FLAG) clone OTI4C5 (#TA50011, OriGene Technologies). Western blot detection was achieved using anti-ADA2 (#HPA007888, Sigma-Aldrich). (C) Western blot of ADA2 pulled down from the whole cell lysate (WCE) and supernatant (SN) of healthy control human monocyte-derived macrophages using anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam) for immunoprecipitation and detection with VeriBlot. All samples were prepared in four technical replicates. Three replicates were analyzed by mass spectrometry, the fourth replicate is shown on western blot. IgG samples represent isotype controls performed in parallel. The western blot is representative of n=5 (3 healthy controls; 2 patients).

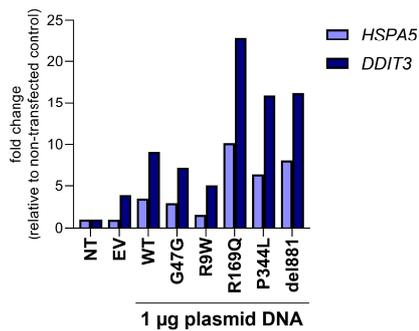
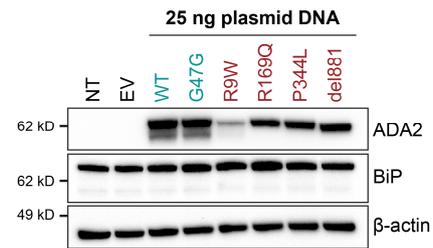
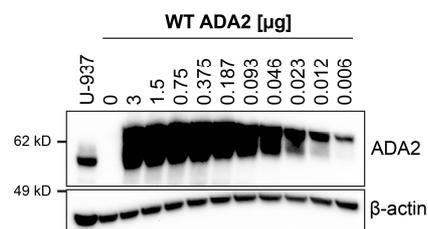
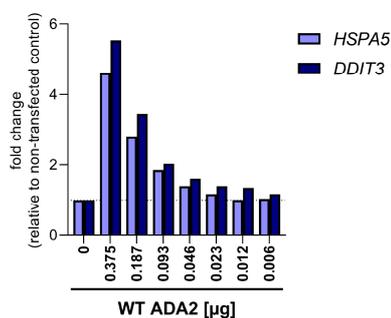
A HEK293T**C HEK293T****B HEK293T**

Fig. S8: ER stress after overexpression of pathogenic *ADA2* variants. ER stress (A) ER stress response after overexpression of pathogenic *ADA2* variants. HEK293T cells were transfected with 1 µg of plasmid DNA to overexpress wild-type (WT) *ADA2* and different pathogenic variants. The sham variant p.G47G was included as an internal control. Samples were taken 48h after transfection and ER stress was measured by mRNA expression of *HSPA5* and *DDIT3* normalized to *GAPDH*. Expression is depicted as $2^{-\Delta\Delta C_t}$ relative to non-transfected control (NT). Bar graphs show values of n=1 experiment. (B) Titration of plasmid DNA used for overexpression of *ADA2*. HEK293T cells were transfected with the indicated amounts of DNA to overexpress wild-type (WT) *ADA2*. Samples were taken 48h after transfection and ER stress was measured by mRNA expression of *HSPA5* and *DDIT3* normalized to *GAPDH*. Expression is depicted as $2^{-\Delta\Delta C_t}$ relative to the non-transfected control (0 µg). Bar graphs show values of n=1 experiment. (C) Western blot corresponding to the dot plots shown in Fig. 5B. ER stress response in HEK293T cells transfected with 25 ng wild-type (WT) *ADA2* and different pathogenic variants. Protein expression of BiP was determined 48h after transfection. The western blot is representative of n=6 independent experiments. *ADA2* was detected by anti-*ADA2* antibody clone EPR25430-131 (#ab288296; abcam).

CD14+ monocytes

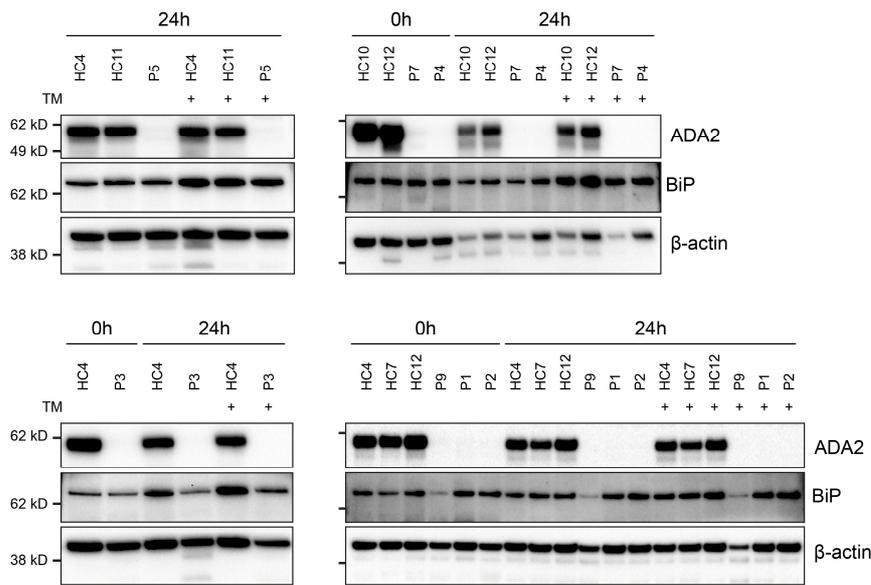


Fig. S9: BiP expression in CD14+ monocytes. The depicted western blots correspond to the dot plots shown in Fig. 5C. ER stress response was evaluated by protein expression of BiP in whole cell lysates of CD14+ monocytes from healthy controls (HC) and DADA2 patients (P) at baseline or after 24h incubation with or without 1 $\mu\text{g}/\text{mL}$ tunicamycin (TM). ADA2 was detected by anti-ADA2 antibody clone EPR25430-131 (#ab288296; abcam).

U-937

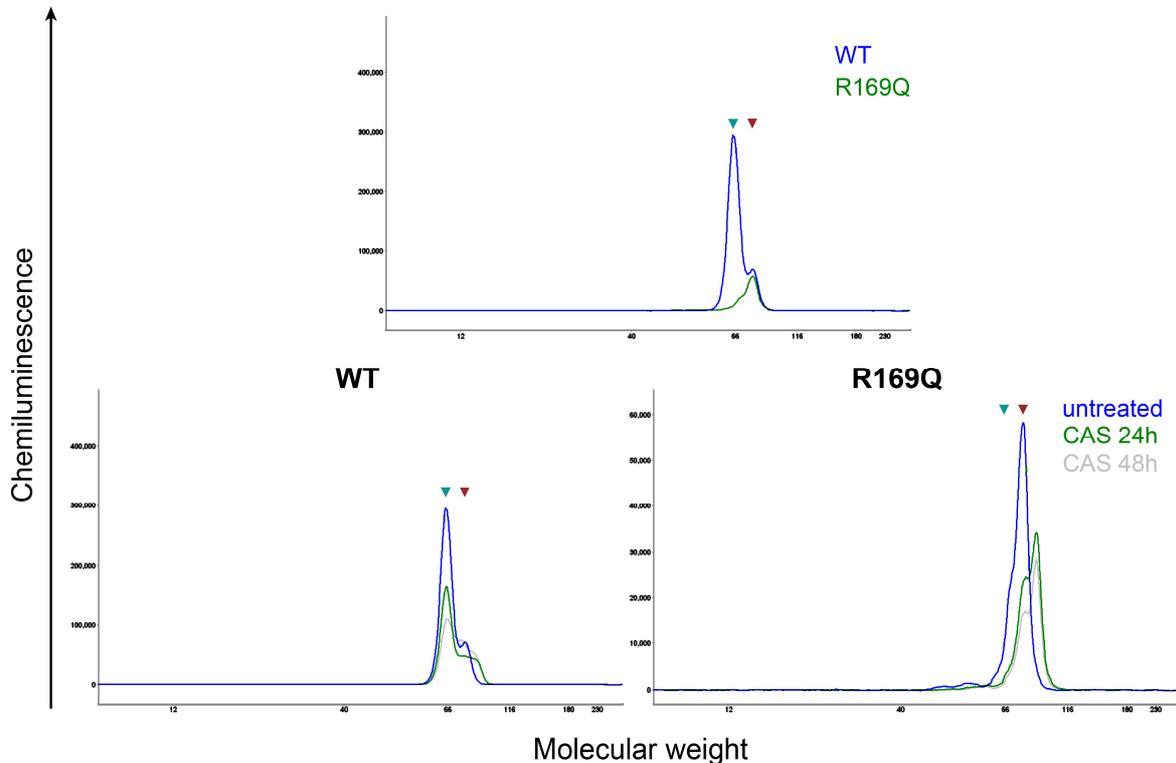


Fig. S10: Molecular weight of ADA2 after inhibition of ER α -glucosidases I and II. ADA2 protein was measured by Simple Western in ADA2^{WT/WT} and ADA2^{-/-} U-937 cells transduced with

R169Q after 24h or 48h incubation with 100 $\mu\text{g}/\text{mL}$ castanospermine (CAS). The graphs show the signal for ADA2 by molecular weight. The top plot depicts the original graph shown in Fig. 7C. Triangles indicate HMW-ADA2 (red) and LMW-ADA2 (green).

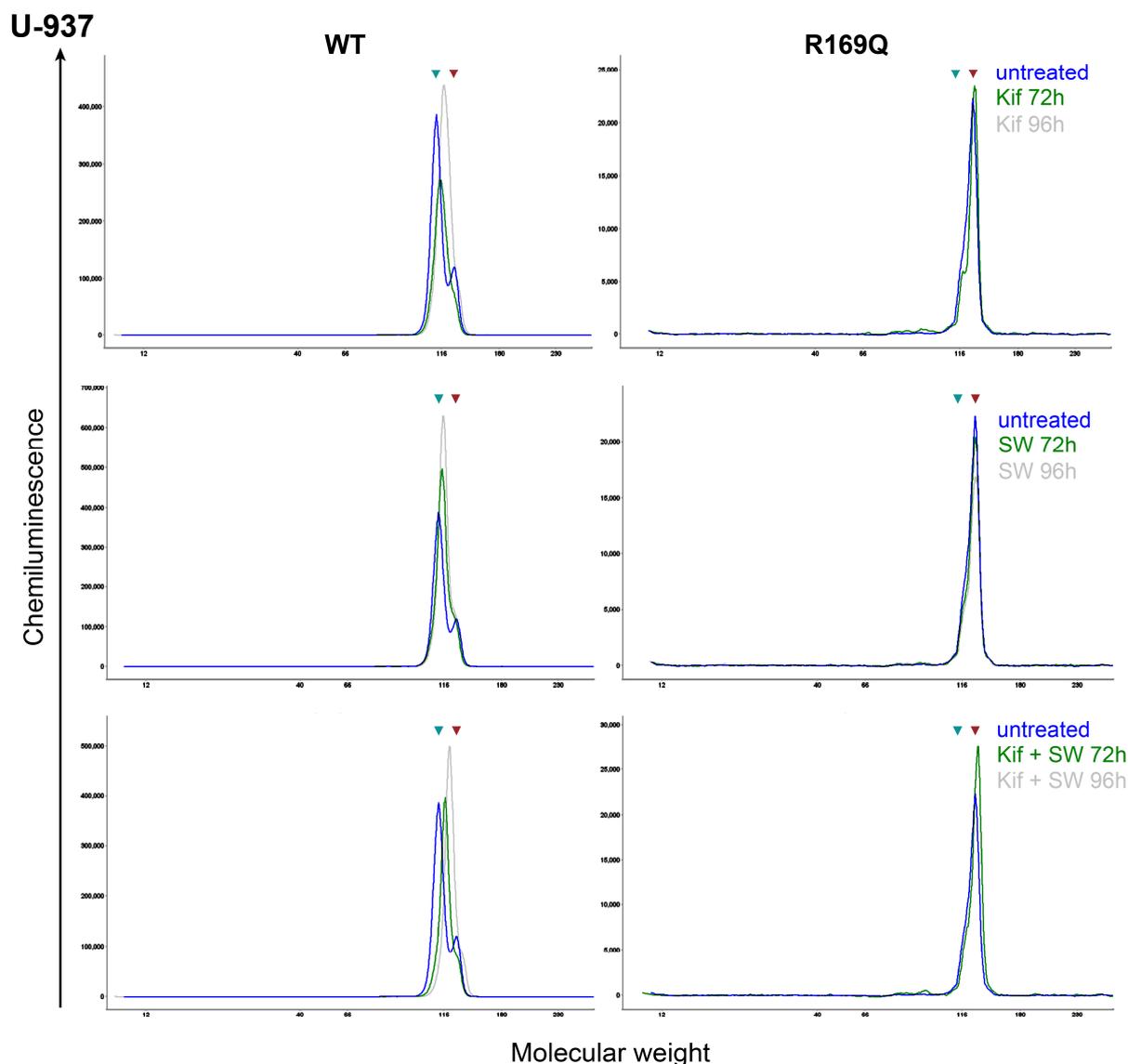


Fig. S11: Molecular weight of ADA2 after inhibition of class I and II α -mannosidases. ADA2 protein was measured by Simple Western in ADA2^{WT/WT} and ADA2^{-/-} U-937 cells transduced with R169Q after 72h or 96h incubation with 375 nM kifunensine (Kif) and/or 10 μM swainsonine (SW). The graphs show the signal for ADA2 by molecular weight. The top left plot depicts the original graph shown in Fig. 7C. Triangles indicate HMW-ADA2 (red) and LMW-ADA2 (green).

Supplementary Tables

ID	Age [years]	Sex	Genotype	
P1	12	M	c.973-2A>G/c.del1240-1442	splice site (intron 6)/del exon 9
P2	14	F	c.973-2A>G/c.del1240-1442	splice site (intron 6)/del exon 9
P3	26	M	c.140G>T/c.del1240-1442	p.G47V/del exon 9
P4	26	M	c.140G>T/c.140G>T	p.G47V/p.G47V
P5	6	F	c.140G>T/c.506G>A	p.G47V/p.R169Q
P6	9	F	c.140G>T/c.506G>A	p.G47V/p.R169Q
P7	32	F	c.973-2A>G/c.506G>A	splice site (intron 6)/p.R169Q
P8	16	M	c.139G>A/c.139G>A	p.G47R/p.G47R
P9	10	F	c.973-2A>G/c.973-2A>G	splice site (intron 6)/splice site (intron 6)
PA	4	M	c.881+1G>A/c.881+1G>A	splice site (intron 5)/splice site (intron 5)
PB	47	F	c.1358A>G/not reported	p.Y453C/p.G450Afs15X
PC	5	F	c.139 G>A/c.139 G>A	p.G47R/p.G47R
CR1	39	F	WT/c. del1240-1442	WT/del exon 9
CR3	42	M	WT/c.506G>A	WT/p.R169Q
CRA	56	F	WT/c.973-2A>G	WT/splice site (intron 6)
CRB	47	F	WT/c.506G>A	WT/p.R169Q
CRC	51	M	WT/c.140G>T	WT/p.G47V
CRD	48	F	WT/c.del1240-1442	WT/del exon 9
CRE	46	M	WT/c.140G>T	WT/p.G47V
CRF	45	F	WT/c.140G>T	WT/p.G47V
CRG	62	F	WT/c.881+1G>A	WT/intron 5: splice site

Table S2: Cohort included in the SomaScan Assay. DADA2 patients (P) and heterozygous carriers (CR) were confirmed by sequencing of the *ADA2* gene.

Legend: Supplementary Table S1

Table S1: Mass spectrometric analysis of top enriched proteins co-immunoprecipitated with ADA2 from HEK293T cells transfected with WT ADA2 or the variant p.R169Q. Fold changes indicate pull-down by anti-DDK antibody targeting tagged ADA2 compared to isotype control.

Appendix I: ADA2 expression and activity in a patient with PMM2-CDG

Since our data highlight the crucial role of N-glycosylation in ensuring successful folding and secretion of ADA2, we analyzed ADA2 expression in a patient with a mild form of *PMM2*-congenital disorder of glycosylation. *PMM2* encodes phosphomannomutase 2, an enzyme involved in the generation of mannose-1-phosphate – a building block required in the early steps of glycan synthesis (42). ADA2 expression and secretion in CD14+ monocytes and HMDM were equal to HC and no differences in molecular weight were noted, indicating unaffected ADA2 glycosylation (Fig. A1). This might however be due to the patient's mild phenotype and residual *PMM2* activity. The patient had normal serum ADA2 enzyme activity (Fig. A1).



Fig. A1: ADA2 expression and activity in PMM2-CDG. *Left:* ADA2 protein expression by western blot in monocyte and HMDM whole cell lysates as well as HMDM supernatants from healthy controls (HC), DADA2 heterozygous carriers (CR), DADA2 patient P4 and a patient with *PMM2*-congenital disorder of glycosylation (CDG). *Right:* ADA2 enzyme activity was determined in the serum. Dot plots show median and interquartile range.

Appendix II: Pathogenic ADA2 variants form intracellular dimers

Intracellular dimer formation of ADA2 has previously been reported only for the ADA2 variant p.L351Q (26). We observed that intracellular dimer formation largely correlated with impairment of ADA2 secretion although even variants with residual secretion, p.G47A, p.G47R, p.N127Q and p.P344L, showed increased dimer formation compared to WT. Inhibition of N-glycosylation – but not of the secretory pathway – led to aggregate formation of WT ADA2 (Fig. S5A+B), confirming the role of N-glycosylation in ensuring the structural integrity of ADA2 (12). We also detected intracellular dimers in DADA2 HMDM, although to a lesser extent due to the strongly reduced protein expression of the pathogenic variants (Fig. S5C). Geldanamycin has been described to induce refolding of misfolded proteins by inhibiting HSP90 (43). For ADA2, this treatment did not reduce intracellular aggregate formation or improve protein secretion (Fig. S6A). Instead, we observed an increase in intracellular ADA2 dimers upon inhibition of the chaperone. Proteasome inhibition by delanzomib also increased intracellular ADA2 aggregates supporting the hypothesis

that they are formed by misfolded ADA2 protein (Fig. S6A). These findings indicate that impaired folding due to mutations or defective N-glycosylation promotes intracellular aggregate formation of misfolded ADA2.

To elucidate whether these aggregates actively disturb ADA2 secretion or form as a result of impaired protein transport, we co-transfected HEK293T cells with varying amounts of WT and mutant ADA2. We found that the presence of the pathogenic variant p.R169Q caused dimer formation and impaired secretion of WT ADA2 overproportionally to the transfected ratios of WT and mutant (Fig. S6B), indicating that aggregate formation may indeed actively disturb ADA2 secretion. In line with this finding, we also observed that ADA2 serum protein levels of heterozygous carriers were in fact only 25% of healthy control (Fig. S6C), while intracellular protein levels represented 75% of HC in CD14+ monocytes (Fig. S6D). Thus, intracellular aggregate formation of pathogenic ADA2 protein variants appears to contribute to impaired secretion of mutant ADA2, at least for the tested variant p.R169Q.