Proximity proteomics reveals UCH-L1 as an essential regulator of NLRP3 mediated IL-1β production in human macrophages and microglia

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Running title: UCH-L1 regulates NLRP3 inflammasome & IL-1β production

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Key words: Deubiquitylase, DUB, IL-1β, inflammasome, NLRP3, protease inhibitor, ubiquitin, UCH-L1

ABSTRACT (148 words)

Activation of the NACHT, LRR family pyrin domain containing 3 (NLRP3) inflammasome complex is an essential innate immune signalling mechanism. To reveal how NLRP3 inflammasome assembly and activation are controlled, in particular by components of the ubiquitin system, proximity labelling, affinity purification and RNAi screening approaches were performed. Our study provides an intricate time-resolved molecular map of different phases of NLRP3 inflammasome activation. We discovered that ubiquitin C-terminal hydrolase 1 (UCH-L1) interacts with the NACHT domain of NLRP3, and downregulation of UCH-L1 decreases pro-IL-1β levels. UCH-L1 chemical inhibition with small molecules interfered with NLRP3 puncta formation and ASC oligomerization, leading to altered IL-1β cleavage and secretion, particularly in microglia cells, which exhibited elevated UCH-L1 expression as compared to monocytes/macrophages. Altogether, we profiled NLRP3 inflammasome activation dynamics and highlight UCH-L1 as an important modulator of NLRP3-mediated IL-1β production, suggesting that a pharmacological inhibitor of UCH-L1 may decrease inflammation-associated pathologies.

INTRODUCTION

Innate immunity reflects a cornerstone of defence systems in living organisms which integrates with adaptive immune responses in the case of higher eukaryotes (Paludan et al., 2021). A major hallmark is the activation of inflammasome complexes, composed of ASCs and NLRs, such as NACHT, LRR family pyrin domain containing 3 (NLRP3) (Martinon et al., 2002) involving cleavage of pro-IL1β by caspase-1 (Gross et al., 2012). External stimuli including lipopolysaccharide (LPS) as the first, and nigericin, monosodium urate (MSU) monohydrate, Alum, ATP or cholesterol crystals as the second signal lead to the assembly and activation of the NLRP3 inflammasome complex, resulting in inflammatory responses (Shao et al., 2015; Swanson et al., 2019). Assembly of this supramolecular structure, featuring NLRP3 and ASC, is further regulated by associated factors, such as NEK7 (He et al., 2016), as well as specific posttranslational modifications including phosphorylation and ubiquitylation (Akther et al., 2021; Liang et al., 2021; Zangiabadi and Abdul-Sater, 2022). In addition to the kinases RIPK3 (Lawlor et al., 2015), FYN (Panicker et al., 2019) and ubiquitin ligases TRIM31(Song et al., 2016), gp78/Insig-1 (Xu et al., 2022), MARCH7 (Yan et al., 2015), HUWE1 (Guo et al., 2020), RNF25 and Cbl (Tang et al., 2020), deubiquitylases (DUBs) have been shown to modulate ubiquitylation of components of the inflammasome often in the context of specific diseases. More specifically, ubiquitylation and deubiquitylation of NLRP3 and IL-1β (Vijayaraj et al., 2021) have been reported to reflect critical checkpoints of NLRP3 inflammasome complex assembly, activation and signalling (Lopez-Castejon, 2020; Lopez-Castejon and Edelmann, 2016; Lopez-Castejon et al., 2013). Ubiquitin protease 7 (USP7) and 47 (USP47) inhibition resulted in reduced inflammasome activation

(Palazon-Riquelme et al., 2018). In addition, in mice, BRCC3, a DUB reflecting a component of the BRISC complex, was shown to deubiquitylate NLRP3, thereby regulating inflammasome activity (Py et al., 2013). Furthermore, the ubiquitin-specific proteases USP9X (Xiang et al., 2023), USP14 (Hai et al., 2022) and UCHL5 (Kummari et al., 2015; Ramachandran et al., 2021) have been proposed to deubiquitylate NLRP3 and thereby modulate inflammasome activation in alveolar epithelial cells (AECs) and annulus fibrosus cells derived from patients with intervertebral disc degeneration, respectively. In addition, UAF1 deubiquitylase complexes also promote NLRP3 inflammasome expression (Song et al., 2020) and factors such as ABRO1 regulate activation through NLRP3 deubiquitylation (Ren et al., 2019). Together, these findings suggest a complex interplay between ubiquitylation and deubiquitylation mechanisms that modify components of the inflammasome complex, although the hierarchical order and sequence of events are less clear.

To study in more detail the ubiquitin-mediated regulation of NLRP3 inflammasome, we have combined ascorbic acid peroxidase 2 (APEX2)-based proximity labelling, affinity purification and RNAi screening to discover molecular factors involved in the primary and secondary signals of NLRP3 inflammasome activation and assembly. We demonstrate that UCH-L1 interacts with the NACHT domain of NLRP3 and thereby interferes with ASC and NLRP3 assembly. Furthermore, ubiquitin C-terminal hydrolase 1 (UCH-L1) knockdown or chemical inhibition interferes with IL-1β production, particularly in microglia cells that exhibited elevated UCH-L1 expression as compared to peripheral monocytes or macrophages.

RESULTS

NLRP3-APEX2 captures the NLRP3 molecular proximity network

As the N-terminus NLRP3 PYD domain has been reported to be responsible for initiating NLRP3 oligomerization, we inserted APEX2 at the C-terminus (LRR domain) of NLRP3 including a flexible linker between these domains and a flag epitope at the N-terminus (**Figure 1A**). To avoid unstable expression and mis-localization during transient transfection, we generated a NLRP3-APEX2 HEK293 stable cell line using the Flp-In T-REx system, allowing dose-dependent control of expression with tetracycline (**Figures S1A, B**). Fluorescence microscopy confirmed that both flag-NLRP3 and flag-NLRP3-APEX2 can form puncta in the perinuclear areas in response to nigericin stimulation, while the fusion proteins are diffused across the cytosol in the resting state (**Figures S1C, D**). ASC oligomerization assays with co-expression of adaptor protein ASC in stable cell lines proved that both flag-NLRP3 and flag-NLRP3-APEX2 are competent to recruit ASC and induce its polymerization upon stimulation with nigericin (**Figures S2A, B**). Taken together, these results indicate that APEX2 tagging

fused to the C-terminus of NLRP3 does not impede its ability to sense stimuli, particularly nigericin induced potassium efflux, and recruit ASC to form oligomers.

We next investigated whether biotin labelling profiles are spatially restricted to the fusion bait. Live HEK293 cells expressing NLRP3-APEX2 were treated with or without nigericin. Subsequently, cells were treated with H_2O_2 in the presence of biotin-phenol, followed by rapid quenching, methanol fixation and staining (**Figure 1B**). In contrast to the native control without H_2O_2 treatment, we observed robust biotinylation by streptavidin-Alexa Fluor 488 imaging. Under unstimulated conditions, NLRP3-APEX2 was diffused across the cytosol, with biotinylated proteins following similar distribution pattern. Upon nigericin treatment, NLRP3-APEX2 oligomerized to form puncta close to the nucleus, and we also observed specks formed by biotin labelled proteins (**Figure 1C**). This partially suggests the spatial specificity of NLRP3-APEX2 induced biotinylation.

The ability of APEX2 to efficiently label and capture the NLPR3 "proximitome" was assessed using streptavidin immunoblotting and Coomassie blue staining, confirming the robust biotin labelling activity of APEX2 fused to NLRP3 (**Figure 1D, Figure S3B**). Initially, we investigated whether APEX2 is competent to capture the known NLRP3-interacting protein-NIMA-related kinase 7 (NEK7). In contrast to the unlabelled negative control, we found that NLRP3-APEX2 specifically biotinylated NEK7. Additionally, a substantial increase in NEK7 protein sequestration was observed in association with activated NLRP3 (**Figure 1D**).

Given the rapid kinetic properties of APEX2 proximity labelling, we next sought to map the spatiotemporally resolved "proximitome" of NLRP3 upon stimulation by nigericin across various time intervals. Following mass spectrometric analysis, 3,478 proteins in total were detected across the DMSO control and nigericin treatment for 30 min, 60 min and 90 min. To maximize the signal-to-noise ratio and discriminate the bona fide interacting partners from the background contaminants, Bayesian model-based algorithm -SAINTexpress (Teo et al., 2014) was applied, yielding 800-1,100 high confidence NLRP3 proximal proteins (**Figures 2A-B, Figure S3A**). Unsupervised principal component analysis (PCA) showed the clustering and patterns of different protein subgroups (**Figure 2C**). High correlation was observed between the three biological repeats within each group confirming the low technical variation in this experiment (**Figure S3C**).

To assess the specificity of the NLRP3 proximity proteome, we next sought to check if the known NLRP3 interactors or regulators are biotinylated and captured by APEX2. SAINT probability scoring and simpler fold-change (FC) were calculated by the comparison of prey protein abundance between the bait and negative beads control. Previously reported 83 human NLRP3 interactors, retrieved from public interaction repository BioGrid (https://thebiogrid.org/, version 4.4.209) and the literature (**Table S1**) (Oughtred et al., 2021; Oughtred et al., 2019; Stark et al., 2006) were mapped and plotted with stringent thresholds (SAINT probability > 0.8, FC-A ≥ 1). After filtering, 14 known NLRP3 interactors

(16%) were discerned as high-confidence hits within the proximal proteomic data (**Figures 2D-H, Figure S4**) confirming the enrichment of NLRP3-associated proteins.

Temporal tracking of the NLRP3 proximity proteome

To gain mechanistic and functional insights into the NLRP3 proximity proteome following nigericin stimulation at various intervals, we conducted a pathway enrichment analysis (**Figure 2I**). In terms of gene ontology-biological process (GO term-BP) enrichment analysis, 13 shared terms, including viral translation, apoptotic cell death regulation, stress granule assembly and mitosis-associated processes, were significantly enriched across all four groups. In contrast to the unstimulated control, pathways related to "protein kinase D signalling" and "mRNA binding regulation" were uniquely present after nigericin-exposure, consistent with prior observations (Zhang et al., 2017). Intriguingly, the enrichment of "cellular response to arsenite ion" and "regulation of stress granule disassembly" terms suggest a potential functional association between inflammasome and stress granule formation. This connection was substantiated by previous reports of the stress granule protein DDX3X as a pivotal mediator, modulating between apoptotic ASC speck formation and pro-survival stress signalling (Fox and Man, 2019; Samir et al., 2019). In terms of GO term-molecular function (GO term-MF) enrichment analysis, "GTP-dependent protein binding" and "1-phosphatidylinostitol binding" were significantly enriched solely in the nigericin-60 min and nigericin-90 min groups, suggesting that nigericin treatment might induce the recruitment of proteins associated with the above two biological functions (**Figures S5A-C**). GO term-cellular components (CC) analysis identified 16 common subcellular compartments or complexes, including COPII vesicle coat, ESCRT-0 complex, and chaperone complex. Notably, three endocytic recycling-associated complexes, including the endosome-associated recycling protein (EARP) complex, endosome to plasma transport vesicle and Golgi-associated retrograde protein (GARP) complex, were significantly enriched solely in nigericin-60 min and nigericn-90 min groups, suggesting that the NLRP3 complex may translocate to trans-Golgi network (TGN) or endosome upon stimulation (**Figure S5D**). Similarly, the compartments enrichment analysis identified another four complexes, including Dcp1-Dcp2, signal recognition receptor, MAD1 and the cohesin I complex, which were only captured and enriched in the last two timepoints of nigericin treatment (**Figures S6A-E**). We also performed tissue enrichment analysis, and not surprisingly, poor tissue specificity was observed in either unstimulated or stimulated groups (**Figure S5E**). To further explore the physiological and pathological biological processes, a reactome pathway analysis was performed on the proximity proteome. The top 20 significantly enriched pathways are shown as in **Figure S6F**. Notably, several innate immunity and virus infection associated pathways, such as interleukin-6 signalling, MHC class I complex, and PP2A-mediated dephosphorylation of key metabolic factors, were identified and enriched. Selected gene sets involved in the above pathways are plotted in **Figures S6G-H**.

Localization analysis reveals translocation of NLRP3 upon stimulation

As the subcellular relocalization of NLRP3 and its underlying mechanism remain controversial (Hamilton and Anand, 2019), we performed the localization analysis to assess the spatial properties of NLRP3 inflammasome during complex assembly. After the filtering, normalization, and imputation, we compared the relative abundance of biotinylated proteins in the unstimulated (DMSO) and stimulated states (nigericin-90 min). The resulting volcano plot (**Figure S3F**) shows 103 proteins enriched in the right quadrant for the stimulated state, while 104 proteins enriched in the left quadrant for the resting state, with relatively stringent cut-off for fold change and false discovery rate (log2[FC]≥0.5 or ≤-0.5, FDR≤ 0.05). GO term-CC enrichment analysis was performed on the differentially enriched proteins in either condition. The proximity proteome under stimulated condition showed high spatial specificity for Golgi apparatus, with 24 proteins falling into the GO-CC term category-Golgi membranes (**Figures S3D-F, Table S2**). This is consistent with the previous observation that NLRP3 is activated and recruited to dispersed trans-Golgi networks (dTGN) in response to diverse stimuli (Bordon, 2019; Chen and Chen, 2018).

Mapping stimulation-dependent NLRP3 interaction alterations using APEX2-PL and AP-MS

Modulation of the NLRP3 local milieu during the activation process was further examined using an integrated approach that combined NLRP3 APEX2 proximity labelling (PL) and affinity purification mass spectrometry (AP-MS) (**Figure 3A**). For APEX2 PL-MS, the SAINTexpress analysis identified 848 (DMSO) and 833 (Nig-90min) proximal proteins, while 484 (DMSO) and 366 potential interacting proteins were captured using the AP-MS method (**Figures 3A, B, Figures S7A-H**). There was relatively small number of overlapping proteins between APEX2 and AP-MS possibly as a result of the complementary nature of these two techniques (**Figures 3C, D**) and consistent with previous studies comparing BioID and AP-MS (Lambert et al., 2015). Additionally, we sought to compare the proximal proteome of NLRP3 under both resting and activation states. Following a rigorous filtration, seven known NLRP3-interacting proteins were differentially enriched. Of these, five proteins (NEK7, MAVS, PRKD1, PRKD2, and PRKD3, highlighted in red) enriched in the right quadrant are established positive regulators of NLRP3. In contrast, EIF2AK2 and LYN (highlighted in green) in the left quadrant are previously characterized inhibitory mediators. Interestingly, we noticed an increased labelling and capture of NLRP3 under stimulated conditions, likely due to oligomerization (**Figure 3E**).

To gain a visual understanding of the interaction networks within the NLRP3 proximity proteome and infer related biological insights, we performed a PPI network analysis. As for the nigericin stimulated condition (**Figure 3F)**, we identified protein complexes including the NLRP3 inflammasome, NEK7, MAVS, the SNARE complex, COPII vesicle coat, the signal recognition particle receptor complex and the protein kinase D signalling complex. **Figure 3G** highlights the interaction networks linked to the unstimulated state, in which the centrosome, the catenin complex, translational initiation factor, calcium dependent serine kinase and the N-end rule proteasomal pathway were significantly enriched.

NLRP3-associated DUBs identified by proximity proteome-guided RNAi screening

The proximity proteome enabled us to capture several ubiquitin system components including 18 DUBs in total (USP9X, USP8, USP5, USP47, USP32, USP24, USP19, USP15, USP14, USP10, USP1, UCH-L1, PAN2, OTULIN, OTUID4, OTUB1, EIF3H and CYLD), which passed the stringent filtering criteria (SAINT probability > 0.8, FC-A ≥ 1). Among the 18 enriched DUBs, 9 were consistently present in all the four groups, and no marked difference was noted between the stimulated and unstimulated states (**Figures 4A-E**). To complement this, we carried out a DUB library RNAi screen in PMA-differentiated THP-1 cells, aiming to assess their influences on NLRP3 inflammasome priming and activation, reflected by IL-1β production. In total, knockdown of 25 DUBs (outliers that surpass mean ± 1 standard deviation, z score ≥1 or ≤-1, **Table S3**) were found to alter IL-1β levels in THP-1 cells treated with LPS or LPS /nigericin (**Figures 4F-G**). A combination of proximity proteomics and RNAi screening revealed 7 DUBs showing effects in both assays (USP9X, USP8, USP14, USP10, UCH-L1, OTUB1 and OTUD4) (**Figure 4H**). USP9X, USP14, and OTUB1 have already been reported to modulate the NLRP3 inflammasome (Hai et al., 2022; Xiang et al., 2023), suggesting additional NLRP3-associated DUBs (USP8, USP10, OTUD4 and UCH-L1).

UCH-L1 interacts with the NACHT domain of NLRP3 and affects IL-1β production

Amongst these DUBs, UCH-L1 consistently affected IL-1β levels and showed high NLRP3 proximity scores. Notably, the knockdown of UCH-L1 did not alter pro-IL-1β mRNA expression, while decreased protein levels and production of the mature form in THP-1 monocytes (**Figures 4I, J**) and human PBMCs (**Figures 4K, L**). Expression of IL-6 and TNFα were also slightly suppressed in the absence of UCH-L1 (**Figure S8B, C**), indicating that UCH-L1 might also play a role in the NF-κB-regulated priming stage. As UCH-L1 is highly abundant in cells associated with the central nervous system, we sought to investigate its role in brain resident immune cells, such as microglia, which also express NLRP3 inflammasome components (**Figure S9**). Similarly to THP-1 and PBMC cells, UCH-L1 deficiency markedly suppressed IL-1β production in the human microglial cell line HMC3 (**Figures 4M, N**).

The NLRP3-UCH-L1 interaction was identified through NLRP3-APEX2 proximity labelling (**Figure 5A**) and confirmed by immunoprecipitation (**Figure 5B**) in HEK293 cells. Notably, the interaction between NLRP3 and UCH-L1 decreases upon nigericin stimulation (**Figure 5B**). To further determine which domain within NLRP3 interacts with UCH-L1, we overexpressed flag-tagged full-length and truncated NLRP3 in HEK293T cells. UCH-L1 interacted with the full-length NLRP3 and NACHT domain of NLRP3 (**Figure 5C**). These results imply that the NACHT domain of NLRP3 directly or

indirectly provides a binding site for UCH-L1. Additionally, immunofluorescence revealed the colocalization of NLRP3 with UCH-L1 in hiPSC-derived microglial cells (**Figures 5D, E**).

UCH-L1 catalytic inhibition abrogates ASC assembly, NLRP3 inflammasome activation and IL-1β processing

To investigate how UCH-L1 modulates IL-1β processing and release, we selected a potent inhibitor-IMP-1711-S (small molecule 27 in patent (Mark Kemp, 2017), **Figure 6A**) (Panyain et al., 2020; Panyain et al., 2021) and tested its effect on NLRP3 inflammasome activation in THP-1, mouse bone marrow-derived macrophages (BMDMs), human PBMCs, and hiPSC-derived microglial cells. Cells were primed with LPS, pre-treated with IMP-1711-S and stimulated with the NLRP3 activator nigericin. We observed that the treatment with IMP-1711-S inhibited the production of IL-1β in a concentration-dependent manner in PMA-differentiated THP-1 (**Figure 6B**) and hiPSC-derived microglia (**Figures 6K, L**). Both the caspase-1 p10 and cleaved IL-1β were reduced in supernatants from IMP-1711-S-treated THP-1 cells (**Figures 6C-E**), suggesting that the inhibitor exerts its effect on the activation of the caspase-1 and IL-1β processing stage. IMP-1711-S potently inhibited the release of lactate dehydrogenase (LDH) and blocked pyroptotic cell death induced by nigericin (**Figures 6F, G**). Additionally, IMP-1711-S treatment did not affect pro-caspase-1 or pro-IL-1β expression in lysates in the priming stage with LPS stimulation (**Figure 6C**). Further investigation of ASC oligomerization in THP-1 cells through crosslinking of the insoluble lysate fraction, revealed that while IMP-1711-S had no impact on ASC expression in cell lysates, it substantially reduced oligomerized ASC levels post LPS and nigericin stimulation (**Figure 6H**). Additionally, in HEK293 cells, IMP-1711-S markedly downregulated the formation of NLRP3 specks in a concentration-dependent manner (**Figures 6I, J**). Our findings underscore that UCH-L1 inhibition impedes NLRP3 inflammasome activation by disrupting NLRP3 assembly and the speck formation, ultimately leading to decreased IL-1β production (**Figure 7**).

DISCUSSION

The NLRP3 inflammasome is central to innate immunity, and a detailed understanding of its molecular dynamics and interactome is highly sought after. Combining APEX2 PL-MS, AP-MS, and siRNA screening, we traced the time-resolved PPI networks local to the NLRP3 inflammasome upon stimulation. This approach offers a resource-rich map for further investigations of the molecular intricacies of NLRP3 inflammasome activation and revealed that UCH-L1 is involved in regulating IL-1β production.

We found that NLRP3 tolerates C-terminally tagged ascorbic acid peroxidase 2 (APEX2), retaining its competence to biotinylate and capture known interacting partners of endogenous NLRP3. However,

certain crucial mediators for NLRP3 activation are absent in the HEK293 cell line model. Despite this limitation, NLRP3-APEX2 captured a plethora of proteins as compared to the negative control (**Fig. 1D**), including NEK7 as a positive control for labelling. NEK7 is an essential regulator for NLRP3 activation downstream of potassium efflux, where it bridges and licenses adjacent NLRP3 to form heterodimer and mediates the complex assembly (He et al., 2016; Sharif et al., 2019).

Deconvolution of the NLRP3-APEX2 proximity proteome

A critical issue related to proximity labelling experiments is discrimination between true interactors and background noise. Proteins captured by NLRP3-APEX2 proximity labelling can be categorized into four groups: Type I-True interacting proteins that physically interact with NLRP3 and or other components of the complex; Type II-Compartmental or proximal proteins in the local milieu of NLRP3 reflecting transient interactions; Type III-non-specific proteins that stick to streptavidin beads; Type IV-physically non-existing proteins detected in error. Type I and type II provide insights into the PPI network and local milieu of NLRP3, but type III and type IV are considered false positives. To establish a high confidence proximity proteome resource for NLRP3, a variety of approaches were used to filter out type III and type IV contaminant proteins. A Bayesian model-based algorithm (SAINTexpress) was applied to analyze the proximity labelling data with a SAINT probability score threshold of 0.8 and fold change $\geq$ 2, yielding 800-1100 proximal proteins per group (**Figures 2C, E-H**). Furthermore, the unstimulated state was used as a control, which helped us distinguishing the differentially regulated interacting partners upon stimulation with nigericin. Even though the SAINTexpress algorithm was used to segregate the true interactors from background contaminants, we still captured more than 800 proteins per biological condition. It is likely that this is due in part to the robust catalytic activity of the APEX2 enzyme, which results in a biotin labelling radius of more than 10 nm in live cells (Qin et al., 2021). With a stringent cut-off, our data identified 103 high-confidence putative NLRP3-associated proteins enriched under activated conditions and 104 proteins enriched under inactivated conditions. Amongst them, we found known interactors, such as NEK7, MAVS, PRKD1/2/3, LYN and EIF2AK2 (**Figure 3E**). We note that, spatial references have been shown to enhance the deconvolution of PL data. However, in our study, this was not applied as NLRP3 resides in multiple compartments and translocates to distinct organelles upon activation (Hamilton and Anand, 2019). By integrating PL data with AP-MS and siRNA screening, we established an alternative method for validating NLRP3-associated proteins.

UCH-L1 is an essential mediator of NLRP3/ASC assembly and IL-1β production

Ubiquitin modification controls multiple elements of the NLPR3 activation process and IL-1β production (Lopez-Castejon, 2020). Through a DUB siRNA screen, we pinpointed four deubiquitylating enzymes, TNFAIP3, CYLD, USP7, and STAMBP, already been linked to IL-1β signalling pathways (**Figure 4G)** (Bednash et al., 2021; Duong et al., 2015; Palazon-Riquelme et al., 2018; Vande Walle et al., 2014; Yang et al., 2020). However, the intricate nature of IL-1β processing, spanning multiple

regulatory stages, poses a challenge to precisely attribute the functional impacts of each DUB. Here, we identified UCH-L1 as a critical modulator of the NLRP3 inflammasome. It is a member of the ubiquitin C-terminal hydrolase family (UCH) family involved in neurodegeneration and cancer metastasis (Ham et al., 2021; Liu et al., 2002; Mondal et al., 2022b). UCH-L1 is highly expressed in the central nervous system, with low expression levels in most other tissues under normal physiology. Dysregulation of UCH-L1 expression and mutations have been implicated in multiple neurodegenerative diseases, such as Parkinson's disease, Huntington's disease, and recessive hereditary spastic paraplegia (SPG79) (Mondal et al., 2022a). Also, several studies linked UCH-L1 to inflammation or innate immunity. For instance, UCH-L1 deficiency markedly reduces the secretion of pro-inflammatory cytokines, notably IL-6 and TNFα (Zhang et al., 2021). LDN57444, an isatin O-acyl oxime small molecule inhibitor, has demonstrated efficacy in ameliorating LPS-induced sepsis in *in vivo* models (Zhang et al., 2021), although other studies have cast doubt on the specificity or efficacy of this compound against UCH-L1 (Panyain et al., 2020). Another study reported that UCH-L1 promoted M1 macrophages polarization via the PI3K/Akt signalling pathway (Huang et al., 2022). Consistent with previous findings, under UCH-L1 knockdown or inhibition, we observed a significant decrease of pro-inflammatory cytokine expression and secretion, including IL-1β, IL-6 and TNFα, in both THP-1 and human primary monocytes, reflecting interference in upstream signalling affecting the priming stage (**Figures 4I-L, Figures S8, S10**). Furthermore, mRNA levels of IL-1β remain unchanged in the absence of UCH-L1, suggesting that UCH-L1 affects IL-1β at the post-transcriptional or protein level (**Figure 4J**). Notably, pharmacological inhibition of UCH-L1 affected ASC/NLRP3 assembly and IL-1β production, which are part of the second inflammasome induction signal (**Figure 6**). Although the small molecule inhibitor IMP-1711-S altered secreted active caspase-1 levels (**Figure 6E**), it does not seem to cross-react or inhibit caspase-1 directly (Panyain et al., 2020). Caspase-1 inhibitors also show a different specificity profile towards TNFα release (Cao et al., 2022). UCH-L1 inhibition interferes with upstream events such as NLRP3 puncta formation and ASC oligomerization (**Figure 6**), although only the latter is phenocopied by UCH-L1 knockdown (**Figure S11**). Exploring further molecular details of UCH-L1 mechanism of action, we confirmed that UCH-L1 is a NLRP3-interacting protein that appears to be present during the activation stage. One possible scenario maybe that UCH-L1, bound to the NACHT domain of NLRP3, acts as a gating/editing factor to control the ubiquitylation status of multiple components, such as NLRP3, ASC, Caspase-1 and IL-1β, required for NLRP3-ASC inflammasome complex assembly (**Figure 7**). Preliminary results indicate that NLRP3's half-live is dependent on the ubiquitin-proteasome pathway (**Figure S12**). NLRP3 associated ubiquitylation appears to be minimally altered by UCH-L1, although NLRP3 protein levels are unaffected (**Figures S12, 13**), suggesting a more structural role for ubiquitin during assembly rather than controlling turnover. Though speculative at this stage, such a putative ubiquitylation editing "gate keeping" process maybe in analogy to the role of UCHL5/UCH37 in the 26S proteasome complex, required for substrate deubiquitylation and ubiquitin

recycling prior to substrate unfolding by the 19S and entry into the 20S for destruction (Song et al., 2021; Vander Linden et al., 2015).

In conclusion, in this study we provide a detailed NLRP3 inflammasome interaction map at different physiological stages. UCH-L1 was identified and validated as an essential regulator involved in NLRP3 inflammasome activation and IL-1β production, affected by UCH-L1 chemical inhibition and phenocopied by UCH-L1 deletion. Our results may have translational implications in exploring pharmacological inhibition of UCH-L1 in neuroinflammation-associated pathology, which could include dementia.

Figure 1

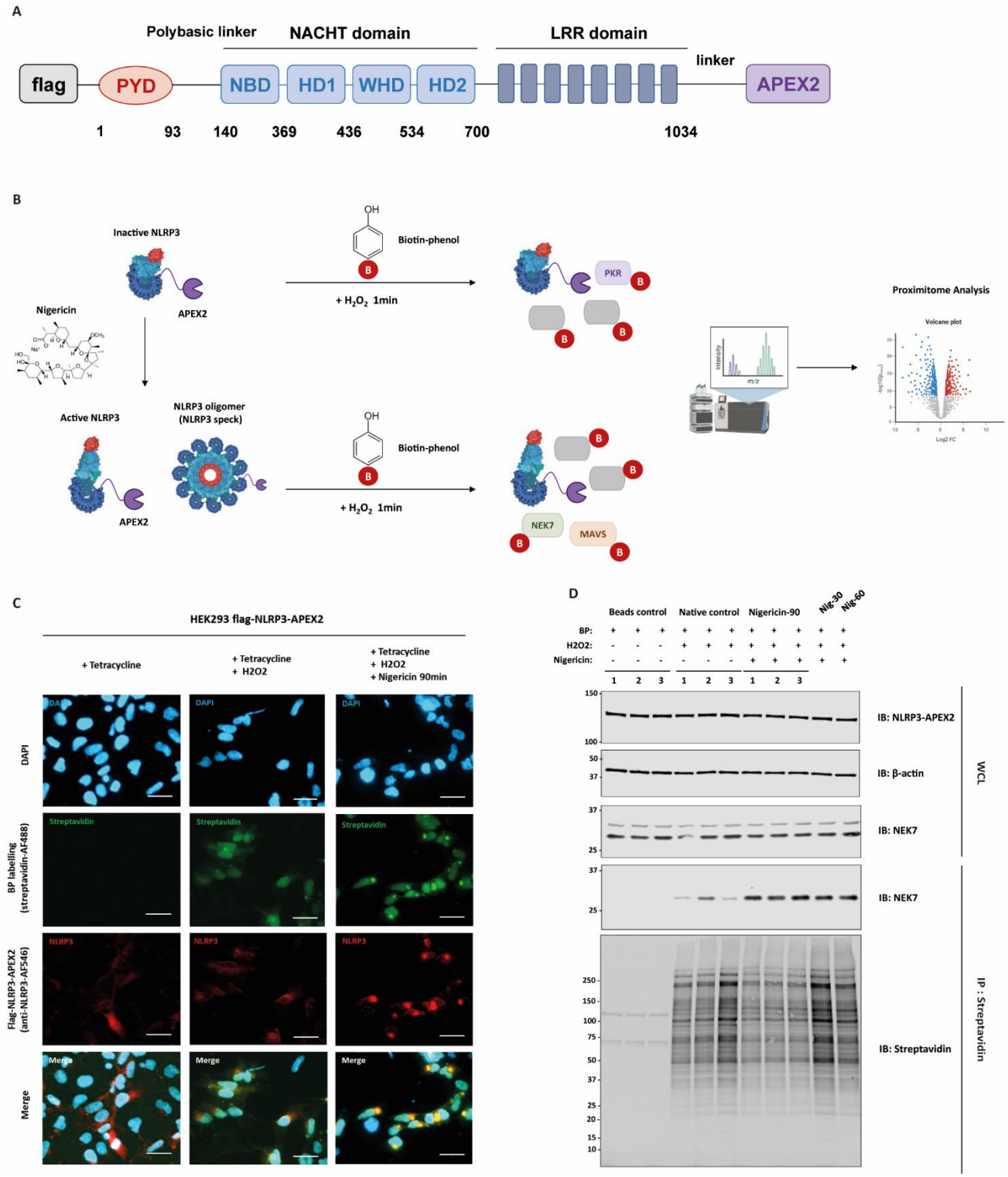

Figure 1 | Proximity proteomic profiling of the NLRP3 inflammasome upon activation

(A) Scheme of APEX2-tagging NLRP3. APEX2 was fused to the C terminus of full-length NLRP3 via a glycine-serine linker. (B) Proximity labelling and mass spectrometric analysis workflow for NLRP3-APEX2. (C) Confocal fluorescence imaging of NLRP3 APEX2 labelling in HEK293 cell lines. NLRP3-APEX2 HEK293 stable cell lines were treated with tetracycline overnight, followed by the treatment

nigericin (10μM) or DMSO (control) for 90 min. Subsequently, cells were incubated with biotin phenol, followed by H_2O_2 as indicated. Afterwards, cells were fixed and stained with streptavidin-Alexa Fluor 488 (AF488) conjugate to visualize biotinylated proteins and anti-NLRP3 antibody to visualize the localization of NLRP3-APEX2. BP, biotinylated proteins. Scale bars, 50 μm. (D) Immunoblot showing the biotinylated NEK7 captured by streptavidin beads in negative control, unstimulated and nigericin-stimulated HEK293 Flp-In T-REx NLRP3-APEX2 cells. Cells were treated with tetracycline overnight, and stimulated with nigericin for 30 min, 60 min, and 90 min before the APEX2 labelling. Whole cell lysates and streptavidin pull-down were collected and blotted for NLRP3 and NEK7.

Figure 2

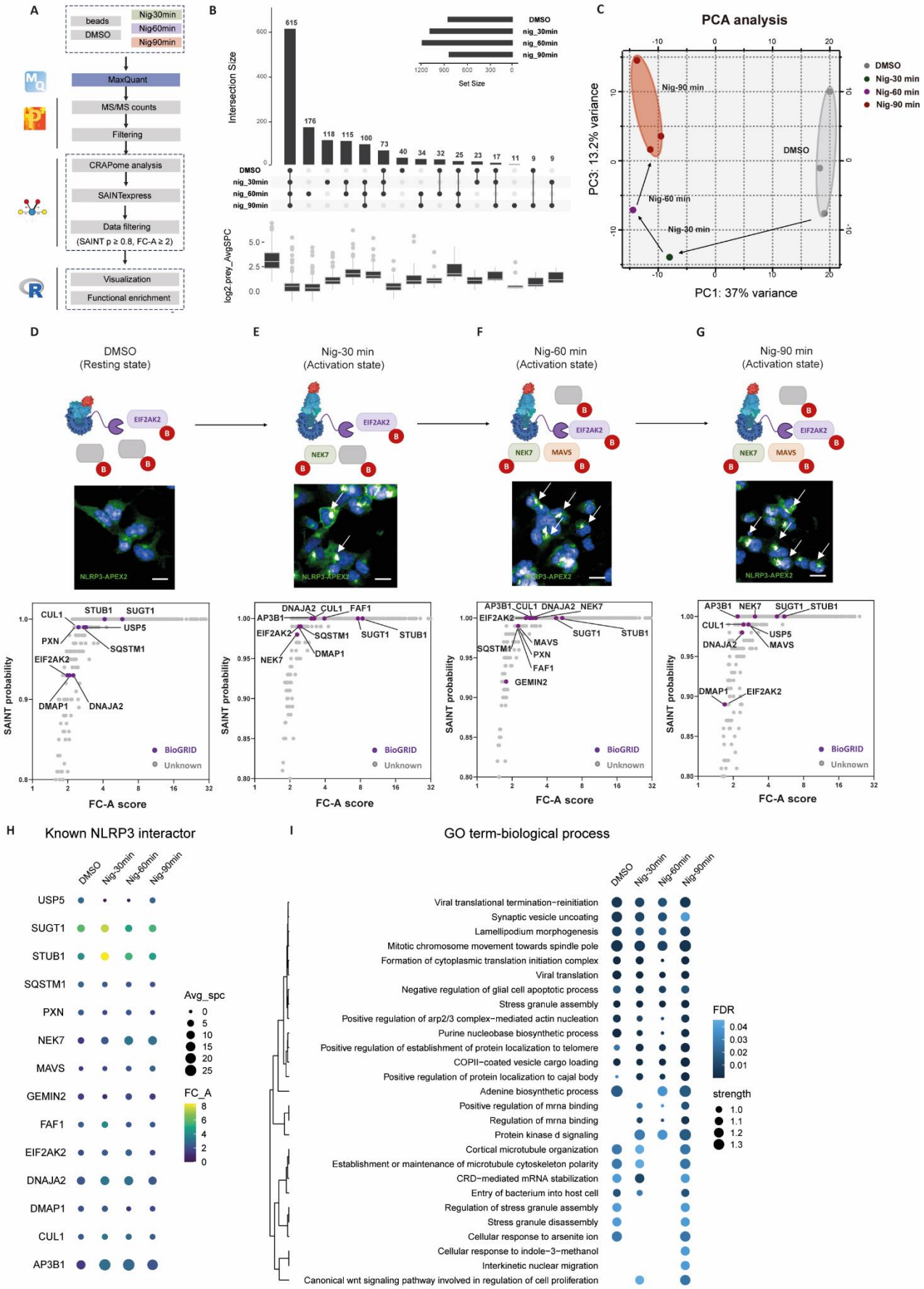
A
beads
DMSO
Nig30min
Nig60min
Nig90min
MaxQuant
MS/MS counts
Filtering
CRAPome analysis
SAINTexpress
Data filtering
(SAINT p ≥ 0.8, FC-A ≥ 2)
Visualization
Functional enrichment
B
Intersection Size
Set Size
log2.prey_AvgSPC
C
PCA analysis
PC3: 13.2% variance
PC1: 37% variance
Nig-90 min
Nig-60 min
Nig-30 min
DMSO
Nig-30 min
Nig-60 min
Nig-90 min
D
DMSO
(Resting state)
E
Nig-30 min
(Activation state)
F
Nig-60 min
(Activation state)
G
Nig-90 min
(Activation state)
EIF2AK2
NEK7
MAVS
NLRP3-APEX2
SAINT probability
FC-A score
BioGRID
Unknown
H
Known NLRP3 interactor
Avg_spc
FC_A
I
GO term-biological process
FDR
strength

Figure 2 | Temporal tracking of the NLRP3 proximity proteome

(A) Schematic illustration of the data analysis for APEX2 PL-MS. (B) The upset plot depicting the intersections of high confidence NLRP3 proximal proteins across samples. The bars in the y axis represent the number of high confidence proximal proteins (SAINT probability score ≥ 0.8, fold change ≥ 2) identified in each sample. The boxplot represents the log2 (average of spectral counts) of the proximal proteins identified in each group. The median, first and third quartiles of the data were displayed as the centre line, the lower and upper boundaries. (C) Principal component analysis of the 8 samples (3 unstimulated DMSO controls, 3 stimulated samples with 90 min treatment of nigericin, 30min nigericin-stimulated samples, and 60 min nigericin-stimulated samples). Each dot represents an individual samples. (D-G) SAINT probability scoring of the proteomic data for NLRP3-APEX2 compared to the negative beads control. Proteins passing the cut off (SAINT probability score ≥ 0.8 and FC-A score ≥ 1) are considered as high confidence proximal proteins for NLRP3. Previously reported NLRP3-interacting proteins (BioGRID, v4.4.213) are coloured in purple. All other proteins are shown in grey. (H) Dot plot of previously reported NLRP3-interacting proteins that are detected in the NLRP3-APEX2 proximitome upon nigericin stimulation for different time points (unstimulated, 30 min, 60 min, and 90 min). Sizes denote the average spectral counts and colours denote the FC-A score. (I) Dot plots displaying the GOBP enrichment analysis of high confidence NLRP3 proximal proteins upon nigericin stimulation for different timepoints as indicated.

427 **Figure 3**

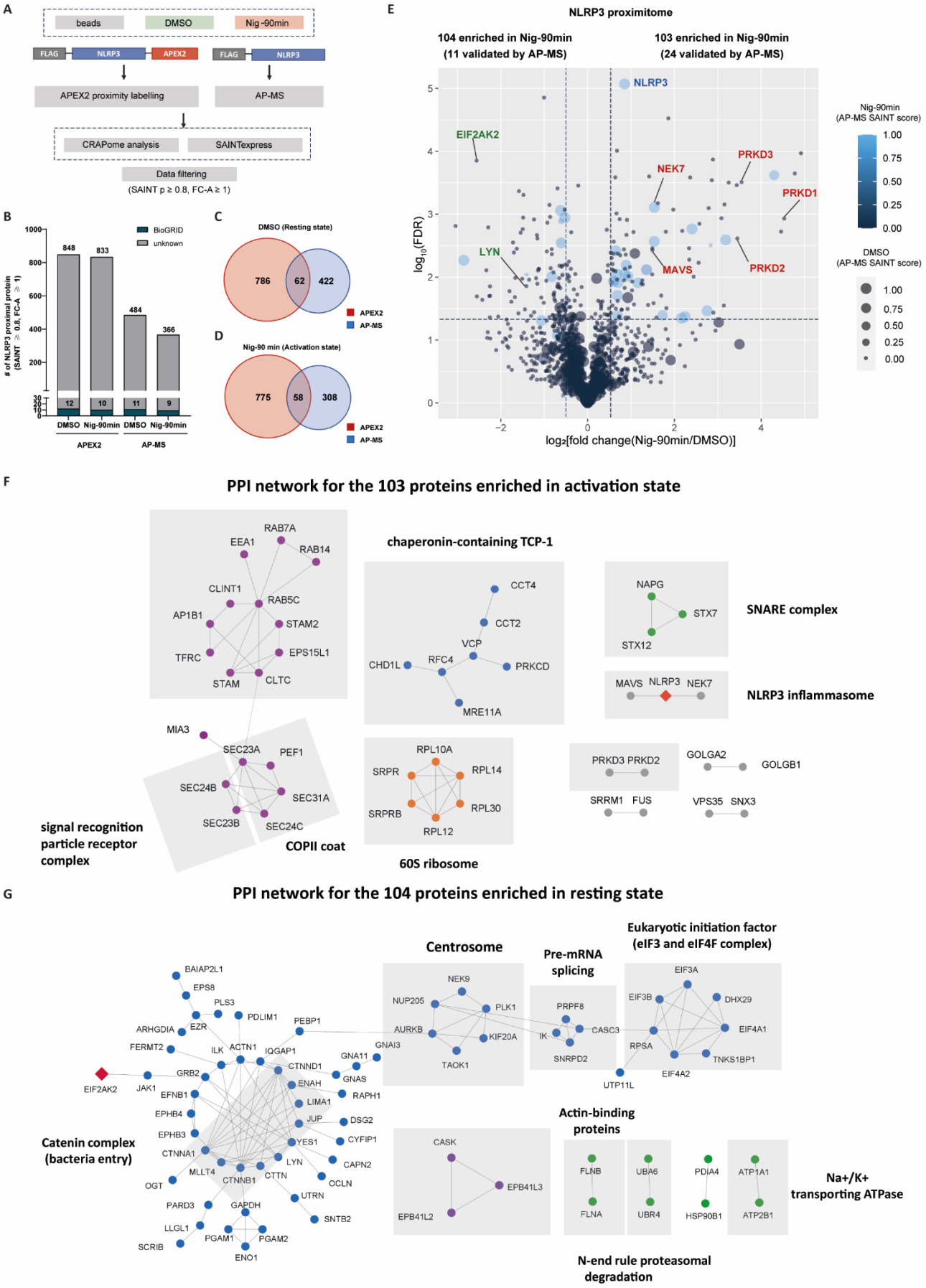

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Figure 3 | Mapping stimulation-dependent NLRP3 PPI alterations using APEX2-PL and AP-MS

(A) Schematic of the integrated approach combining APEX2 proximity labelling and AP-MS. (B) The number of known (BioGRID, blue) and newly discovered NLRP3-interacting proteins (grey) from APEX2 proximity labelling or AP-MS. (C-D) Venn diagrams showing the number of NLRP3 proximal proteins identified in APEX2 proximity labelling or AP-MS. (E) Volcano plot depicting the fold change for relative protein quantification of NLRP3-APEX2 proximity labelling samples between unstimulated (DMSO) and stimulated conditions (nigericin-90min). Data are shown as means of three independent samples. Non-adjusted unpaired two-tailed t-test was applied to the statistical analysis. Sizes denote the SAINT probability score for AP-MS during stimulation (nigericin-90min), and colours reflect the score in its unstimulated condition. (F-G) Protein-protein interaction network for NLRP3-APEX2 proximity labelled proteins enriched after stimulation with nigericin (F) or unstimulated as control (G). MCL algorithm was performed with the PPI scores from STRING database (confidence score threshold = 0.7). Grey lines denote the high-confidence protein-protein interaction curated from Reactome, CORUM and IntAct database. Cluster labels were added manually. n = 3 biological replicates.

Figure 4

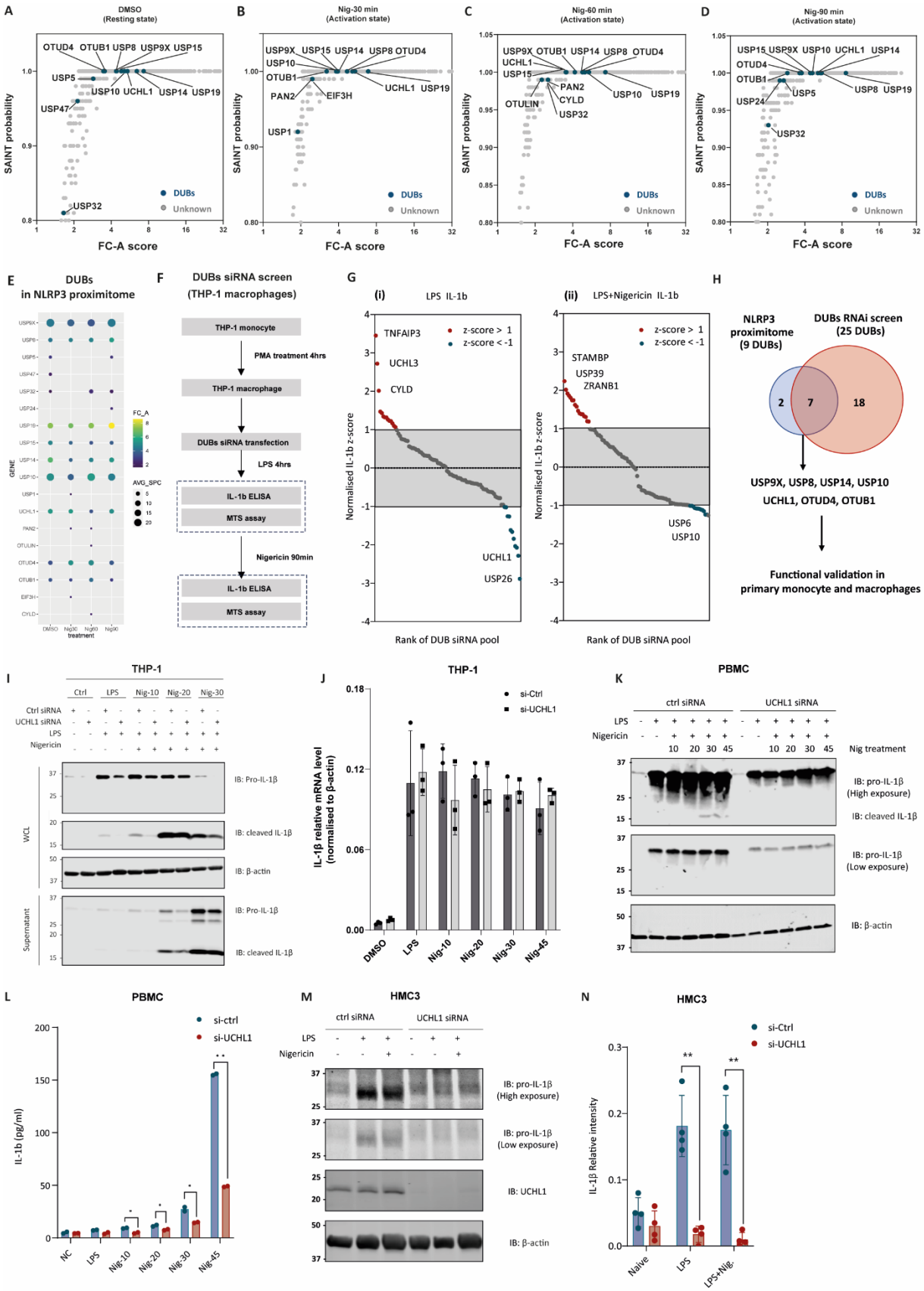

A
DMSO
(Resting state)
SAINT probability
FC-A score
OTUD4 OTUB1 USP8 USP9X USP15
USP5
USP10 UCHL1 USP14 USP19
USP47
USP32
DUBs
Unknown
B
Nig-30 min
(Activation state)
USP9X USP15 USP14 USP8 OTUD4
USP10
OTUB1
PAN2
EIF3H
UCHL1 USP19
USP1
C
Nig-60 min
(Activation state)
USP9X OTUB1 USP14 USP8 OTUD4
UCHL1
USP15
PAN2
CYLD
USP32
OTULIN
USP10 USP19
D
Nig-90 min
(Activation state)
USP15 USP9X USP10 UCHL1 USP14
OTUD4
OTUB1
USP5
USP8 USP19
USP24
USP32
E
DUBs
in NLRP3 proximitome
GENE
FC_A
AVG_SPC
treatment
F
DUBs siRNA screen
(THP-1 macrophages)
THP-1 monocyte
PMA treatment 4hrs
THP-1 macrophage
DUBs siRNA transfection
LPS 4hrs
IL-1b ELISA
MTS assay
Nigericin 90min
IL-1b ELISA
MTS assay
G
(i) LPS IL-1b
z-score > 1
z-score < -1
TNFAIP3
UCHL3
CYLD
UCHL1
USP26
Normalised IL-1b z-score
Rank of DUB siRNA pool
(ii) LPS+Nigericin IL-1b
STAMBP
USP39
ZRANB1
USP6
USP10
H
NLRP3 proximitome (9 DUBs)
DUBs RNAi screen (25 DUBs)
2
7
18
USP9X, USP8, USP14, USP10
UCHL1, OTUD4, OTUB1
Functional validation in primary monocyte and macrophages
I
THP-1
Ctrl
LPS
Nig-10
Nig-20
Nig-30
Ctrl siRNA
UCHL1 siRNA
LPS
Nigericin
WCL
Supernatant
IB: Pro-IL-1β
IB: cleaved IL-1β
IB: β-actin
J
THP-1
si-Ctrl
si-UCHL1
IL-1β relative mRNA level (normalised to β-actin)
DMSO
LPS
Nig-10
Nig-20
Nig-30
Nig-45
K
PBMC
ctrl siRNA
UCHL1 siRNA
LPS
Nigericin
10 20 30 45
Nig treatment
IB: pro-IL-1β (High exposure)
IB: cleaved IL-1β
IB: pro-IL-1β (Low exposure)
IB: β-actin
L
PBMC
si-ctrl
si-UCHL1
IL-1b (pg/ml)
NC
LPS
Nig-10
Nig-20
Nig-30
Nig-45
M
HMC3
ctrl siRNA
UCHL1 siRNA
LPS
Nigericin
IB: pro-IL-1β (High exposure)
IB: pro-IL-1β (Low exposure)
IB: UCHL1
IB: β-actin
N
HMC3
si-Ctrl
si-UCHL1
IL-1β Relative intensity
Naive
LPS
LPS+Nig.

Figure 4 | Proximity proteome-guided RNAi screen identifies deubiquitinases that are involved in IL-1β production.

(A-D) APEX2 proximity labelling identifies DUBs in the local milieu of NLRP3 upon nigericin stimulation. SAINT probability scoring of the proteomic data for NLRP3-APEX2 compared to the negative beads control. Proteins passing the cut off (SAINT probability score ≥ 0.8 and FC-A score ≥ 1) are considered as high confidence proximal proteins for NLRP3. Deubiquitinases are coloured in blue and all other proteins are shown in grey. (E) Dot plots showing the DUBs in the NLRP3 proximity proteome upon nigericin stimulation for different time points as indicated. Sizes denote the average spectral counts and colours denote the FC-A score. (F) Schematic of experimental design for the DUB siRNA library screen. (G) z-scores distribution of the IL-1β in the supernatant for individual siRNAs. The RNAi screen is carried out in PMA-differentiated THP-1 cells upon LPS (i) or LPS+NIG treatment (ii), using ELISA for the measurement of IL-1β in the supernatant. Hits with a z-score of ≤ -1 or ≥1 are chosen as candidates for further validation. (H) Venn diagrams showing the intersection between the hits identified through APEX2-PL and siRNA screen. (I-J) Immunoblot and RT-qPCR analysis of whole cell lysates and supernatants from THP-1 cells transfected with non-targeting control siRNA, or UCH-L1-specific siRNA. Cells were differentiated with PMA and primed with LPS (1 μg/mL) for 4 hours, followed by the treatment of nigericin (10 μM) for different timepoints as indicated. (K-L) PBMCs were transfected with non-targeting control, or UCH-L1-specific siRNA, stimulated with LPS for 4 hours and nigericin for different timepoints as indicated. (K) Immunoblot of cell lysates from PBMCs. (L) Production of IL-1β in the supernatants from PBMCs as measured by ELISA. *$p < 0.05$, **$p < 0.01$ (unpaired two-sided t-test). The results are representative of 3 independent experiments. (M-N) Immunoblot of cell lysates from HMC3 cells transfected with non-targeting control siRNA or UCH-L1-specific siRNA. Cells were primed with LPS (1 μg/mL) for 4 hours, followed by the treatment of nigericin (10 μM) for 45 minutes. (N) IL-1β bands were quantified using GelAnalyzer and normalised using β -actin bands. Error bars show the standard deviation of the mean. **$p < 0.01$ (unpaired two-sided t-test).

Figure 5

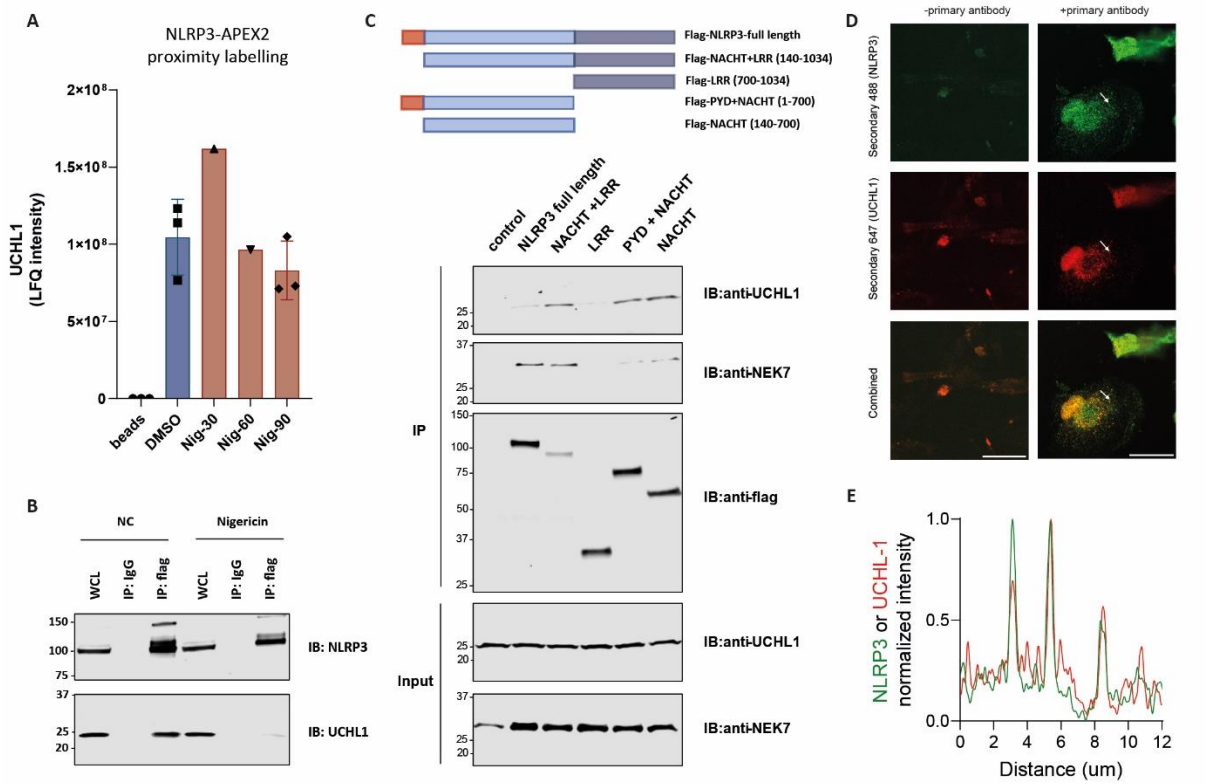


Figure 5 | UCH-L1 interacts with the NACHT domain of NLRP3

(A) The raw LFQ Intensity of UCH-L1 protein identified in the APEX2 proximity labelling performed in this study across all the samples including beads Control, DMSO Control and Nigericin treatment at different time points. Experiment was performed in triplicate except of Nig-30 and Nig-60 where only 1 replicant was performed. (B) Flp-In T-Rex 293 NLRP3-flag stable cells were induced with tetracycline overnight. Afterwards, cells were treated with DMSO or Nigericin for 90 minutes. NLRP3-flag was pulled down using anti-flag antibody or IgG and immunoblotted with indicated antibodies. (C) Schematic representation of the N-terminal Flag-tagged NLRP3 domain constructs: full length NLRP3, NACHT and LRR domain NLRP3 (Flag-NACHT+LRR, amino acids 140-1034), LRR domain of NLRP3 (Flag-LRR, amino acids 700-1034), pyrin and NACHT domain of NLRP3 (Flag-PYD+NACHT, amino acids 1-700), NACHT domain of NLRP3 (Flag-NACHT, amino acids 140-700). HEK293T cells were transfected with the appropriate NLRP3 plasmid using Lipofectamine 3000. Cells were cultured for 24 hours before they were lysed. NLRP3 domain-flag was pulled down using anti-flag antibody and immunoblotted with indicated antibodies. (D) Fluorescence confocal images of hiPSC-derived microglial cells stained for UCHL-1 and NLPR3. UCHL-1 and NLRP3 show colocalization, but only when cells were stained with primary antibodies for UCHL-1 and NLRP3 (right column). No colocalization is shown when cells are not stained with primary antibodies against UCHL-1 and NLRP3 (left column). (E) Line profile showing the min-max normalized intensity of UCHL-1

(red) and NRLP3 (green) along and in the direction of the white arrow indicated in (D). The peaks in normalized intensity indicate colocalization of NLRP3 and UCHL-1 in enriched areas (or ‘specks’) of NLRP3.

Figure 6

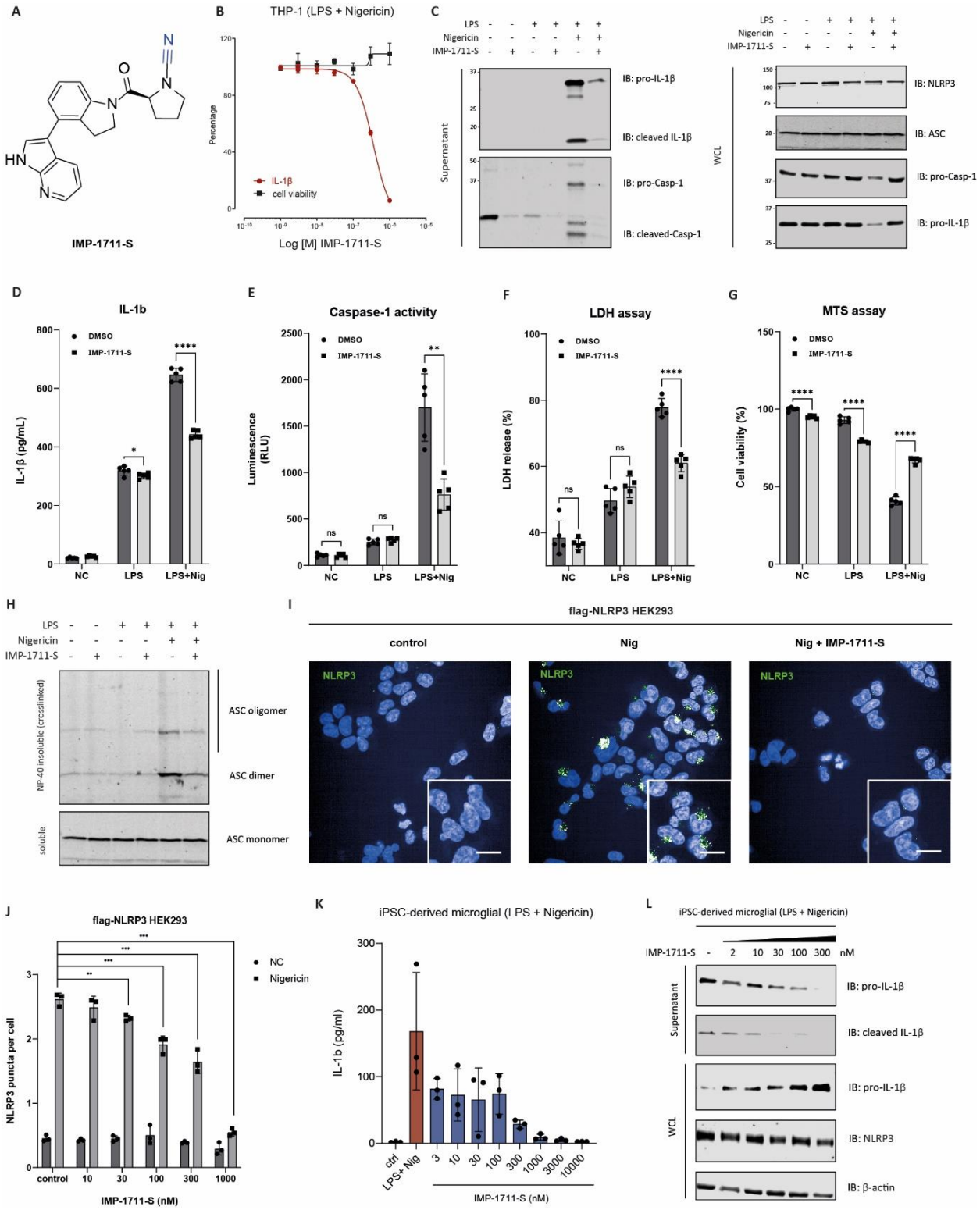
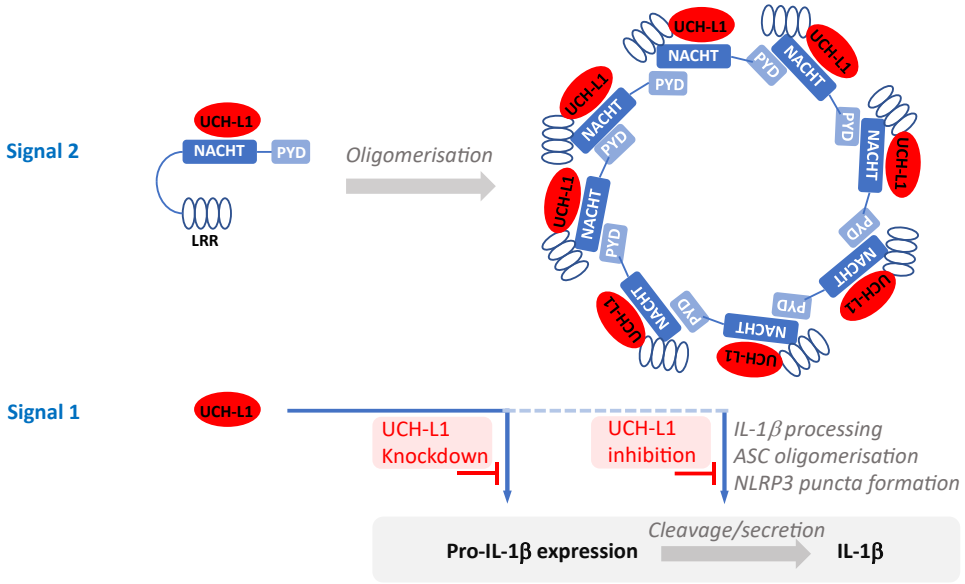

A
IMP-1711-S
B
THP-1 (LPS + Nigericin)
Percentage
IL-1β
cell viability
Log [M] IMP-1711-S
C
LPS
Nigericin
IMP-1711-S
Supernatant
IB: pro-IL-1β
IB: cleaved IL-1β
IB: pro-Casp-1
IB: cleaved-Casp-1
WCL
IB: NLRP3
IB: ASC
IB: pro-Casp-1
IB: pro-IL-1β
D
IL-1b
DMSO
IMP-1711-S
IL-1β (pg/mL)
NC
LPS
LPS+Nig
E
Caspase-1 activity
Luminescence (RLU)
F
LDH assay
LDH release (%)
G
MTS assay
Cell viability (%)
H
NP-40 insoluble (crosslinked)
soluble
ASC oligomer
ASC dimer
ASC monomer
I
flag-NLRP3 HEK293
control
Nig
Nig + IMP-1711-S
NLRP3
J
flag-NLRP3 HEK293
NLRP3 puncta per cell
NC
Nigericin
control
IMP-1711-S (nM)
K
iPSC-derived microglial (LPS + Nigericin)
IL-1b (pg/ml)
ctrl
LPS+ Nig
IMP-1711-S (nM)
L
iPSC-derived microglial (LPS + Nigericin)
IMP-1711-S
nM
Supernatant
IB: pro-IL-1β
IB: cleaved IL-1β
WCL
IB: pro-IL-1β
IB: NLRP3
IB: β-actin

Figure 6 | UCH-L1 inhibitor blocks ASC/NLRP3 assembly and IL-1β production

(A) UCH-L1 inhibitor IMP-1711-S structure. (B) Red, production of IL-1β from PMA-differentiated THP-1 cells stimulated with LPS and nigericin and treated with IMP-1711-S (3-10,000 nM) as measure by ELISA (red). Black, cell viability measured by MTS assay. Data are expressed as mean ± s.d., n = 3 biologically independent samples. (C-G) THP-1 cells were differentiated with PMA and primed with LPS (1 μg/mL) for 4 hours, followed by the treatment of nigericin (10 μM) for 45 minutes. DMSO or IMP-1711-S (0.3 μM) was added at the same time with LPS. (C) Immunoblot analysis of whole cell lysates and supernatants from THP-1 cells treated with DMSO or IMP-1711-S. (D-E) Supernatants were collected for the measurement of IL-1β (D), Caspase-1 activity (E) from 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (unpaired two-sided t-test). Additionally, the cell viability was measured using LDH assay (F) and MTS assay (G). (H) Immunoblot analysis of ASC polymerization upon treatment of DMSO or IMP-1711-S. (I-J) Immunofluorescence imaging of flag-NLRP3 HEK293 stable cells treated with DMSO or IMP-1711-S (300nM), followed by the stimulation with nigericin. Scale bars, 10 μm. (J) Quantitative analysis of the NLRP3 puncta formation upon nigericin treatment in the presence of DMSO or different doses of IMP-1711-S (0.001-1μM) as indicated. **$p < 0.01$, ***$p < 0.001$. (K) IL-1β levels were determined using ELISA from the supernatant of fully differentiated hiPSC-derived microglia. Cells were primed with LPS (1 μg/mL) for 4 hours, followed by the treatment of nigericin (10 μM) for 45 minutes. IMP-1711-S inhibitor was added simultaneously with LPS at different concentrations. (L) Immunoblot analysis of whole cell lysates and supernatants from hiPSC-derived microglia treated with DMSO or different concentrations of IMP-1711-S as indicated.

586 **Figure 7**



587

588 **Figure 7 | Model illustrating multi-functional roles of UCH-L1 in the NLRP3-mediated IL-1β**
589 **expression, processing and production.** UCH-L1 was shown previously to play a role in NF-κB
590 signalling, affecting pro-IL-1β expression (Zhang et al., 2021) in the context of signal 1. UCH-L1
591 pharmacological inhibition interferes with IL-1β production through processes of signal 2 (nigericin)
592 involving ASC oligomerisation, thereby blocking IL-1β processing and secretion.

593

594

STAR★Methods

Key resources table

REAGENTS or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Cleaved IL-1 β (Asp116)	Cell Signalling Technology	CAT# 83186
IL-1β	Cell Signalling Technology	CAT# 12703
Caspase-1	Cell Signalling Technology	CAT# 2225
NLRP3	Cell Signalling Technology	CAT# 15101
ASC(B-3)	Santa Cruz	CAT# sc-514414
β-Actin	Sigma-Aldrich	CAT# A5441
NEK7	Abcam	CAT# ab133514
UCHL1	Santa Cruz	CAT# sc-271639
IRDye® 800CW Goat anti-Rabbit IgG Secondary Antibody	LI-COR	CAT# 926-32211
IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody	LI-COR	CAT# 926-32210
IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody	LI-COR	CAT# 926-68070
IRDye® 680RD Goat anti-Rabbit IgG Secondary Antibody	LI-COR	CAT# 926-68071
Goat anti-Rabbit IgG (H+L) secondary, Alexa Fluor 488-conjugated	Invitrogen	CAT# A-11008
Goat anti-Mouse IgG (H+L) secondary, Alexa Fluor 488-conjugated	Invitrogen	CAT# A-11001
Cell lines and tissue culture reagents		
THP-1	ATCC	TIB-202
HEK293T	ATCC	CRL-3216
Human PBMC	Clinical partners	N/A
KOLF2.1S hiPSC	HipSci project	N/A
Flp-In T-REx 293 Cell Line	Invitrogen	R78007
L-Glutamine (200 mM)	Gibco	25030149
Fetal Bovine Serum	Gibco	10100147
Poly-D-Lysine	Gibco	A3890401
GlutaMAX Supplement	Gibco	35050061
β-Mercaptoethanol	Sigma-Aldrich	M6250
RPMI-1640 Medium	Sigma-Aldrich	R0883

PMA/Phorbol 12-myristate 13-acetate	Sigma-Aldrich	P1585
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140122
Zeocin	InvivoGen	ant-zn-1
blasticidin	InvivoGen	ant-bl-1
hygromycin B	InvivoGen	ant-hg-1
Puromycin Dihydrochloride	Thermo Scientific	A1113803
Dimethyl sulfoxide	Sigma-Aldrich	D8418
Dulbecco's Phosphate Buffered Saline	Sigma-Aldrich	D8537
Chemicals, peptides, and recombinant proteins		
Suberic acid bis(N-hydroxysuccinimide ester)	Sigma-Aldrich	S1885
Sodium deoxycholate	Sigma-Aldrich	D6750
Ultrapure LPS, E. coli 0111:B4	InvivoGen	tlrl-3pelps
Cycloheximide solution	Sigma-Aldrich	C4859-1ML
Bafilomycin A1 from Streptomyces griseus	InvivoGen	B1793
16% Formaldehyde (w/v), Methanol-free	Thermo Scientific	28906
PhosSTOP phosphatase inhibitor	Roche	4906837001
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	11836170001
Trichloroacetic acid	Sigma-Aldrich	T6399
Pierce™ DTT (Dithiothreitol)	Thermo Scientific	20290
Iodoacetamide	Sigma-Aldrich	A3221
Lipofectamine™ 3000 Transfection Reagent	Invitrogen	L3000001
Lipofectamine™ 2000 Transfection Reagent	Invitrogen	11668030
Lipofectamine™ RNAiMAX Transfection Reagent	Invitrogen	13778100
Sequencing-grade modified trypsin	Promega	V5111
Tween-20	Sigma-Aldrich	P1379-1L
Triton X-100	Sigma-Aldrich	T9284-500ML
Bromophenol blue	Sigma-Aldrich	B0126
Biotin	Sigma-Aldrich	B4501
PMSF	G-Biosciences	786-055
Hydrogen peroxide (H_2O_2), 30% (wt/wt)	Sigma-Aldrich	H1009-100ML
Trifluoroacetic acid	Sigma-Aldrich	91707
Formic acid	Sigma-Aldrich	56302

Biotin-phenol		
Dynabeads™ MyOne™ Streptavidin T1	Invitrogen	65602
Sodium ascorbate	Sigma-Aldrich	A7631
Trolox	Sigma-Aldrich	238813-5G
Sodium azide	Sigma-Aldrich	S2002
MG132, Ready Made Solution	Sigma-Aldrich	M7449-1ML
TCEP	Gold Biotechnology	TCEP2
IGEPAL® CA-630	Sigma-Aldrich	I8896
Triton™ X-100	Sigma-Aldrich	X100
Nuclease-Free Water (not DEPC-Treated)	Invitrogen	AM9932
Benzonase® Nuclease	Millipore	E1014
Biochemistry assays		
Caspase-Glo® 1 Inflammasome Assay	Promega	G9951
Pierce™ BCA Protein Assay Kit	Thermo Scientific	23225
InstantBlue® Coomassie Protein Stain (ISB1L)	Abcam	ab119211
CyQUANT™ LDH Cytotoxicity Assay	Invitrogen	C20300
CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS)	Promega	G3582
Dynabeads™ Co-Immunoprecipitation Kit	Invitrogen	14321D
Human IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems	DY201
Human IL-6 DuoSet ELISA	R&D Systems	DY206
Human TNF-alpha DuoSet ELISA	R&D Systems	DY210
LightCycler® 480 SYBR Green I Master	Roche	04707516001
High-Capacity cDNA Reverse Transcription Kit	Thermo Scientific	4368814
RNeasy Mini Kit	Qiagen	74104
Stop Solution 2N Sulfuric Acid	R&D Systems	DY994
Recombinant DNA		
pOG44 Plasmid Vector	ThermoFisher Scientific	CAT# V600520
pcDNA5/FRT/TO GFP	Addgene	CAT# 19444
pcDNA5/FRT/TO APEX2-NLRP3-Flag	Generated in this study (Backbone 19444)	N/A
pEGFP-C2-NLRP3 (ITEM 73955)	Addgene	N/A
pNLRP3 FLAG Full length	Generated in this study (Backbone 73955)	N/A

pNLRP3 NACHT+ LRR- FLAG (140-1035)	Generated in this study (Backbone 73955)	N/A
pNLRP3 LRR only- FLAG (700-1035)	Generated in this study (Backbone 73955)	N/A
pNLRP3 Pyrin + NACHT- FLAG (1-700)	Generated in this study (Backbone 73955)	N/A
pNLRP3 NACHT only- FLAG (140 -700)	Generated in this study (Backbone 73955)	N/A
Software and algorithms		
Prism	GraphPad, version 9	https://www.graphpad.com/scientific-software/prism/
Perseus	Nature methods 13.9 (2016): 731-740. Version 1.6.15.0	https://maxquant.net/perseus/
ImageStudioLite	LI-COR, version 5.2	https://www.licor.com/bio/image-studio-lite/
FiJi	Nature Methods, 9(7), 676–682.	https://imagej.net/software/fiji/
Cytoscape	Genome Research 2003 Nov; 13(11):2498-504	https://cytoscape.org/
ZEN	Carl Zeiss	RRID: SCR_013672
Illustrator	Adobe	https://www.adobe.com/uk/
Photoshop	Adobe	https://www.adobe.com/uk/
MaxQuant	Nat Protoc. 2016 Dec;11(12):2301-2319. Version v1.6.10.43	http://www.maxquant.org

Method details

Cell culture

THP-1 cells were cultured in 10% FBS (Gibco #10500-64) RPMI 1640 (Sigma) supplemented with 2% L-glutamine (G7513, Sigma-Aldrich), 0.05 mM β-mercaptoethanol (Sigma), and antibiotics (penicillin/streptomycin, Gibco). HEK293T cells were cultured in high glucose DMEM, supplemented with 10% FBS, and 2% L-glutamine. Flp-In T-REx 293 cell line (R78007, ThermoFisher) were cultured in high glucose DMEM, supplemented with 10% FBS, 2% L-glutamine, 100 µg/mL Zeocin (InvivoGen, ant-zn-1) and 15 µg/mL blasticidin (InvivoGen, ant-bl-1) at 37 °C in a humidified 5% CO2 atmosphere. The stably transfected Flp-In T-REx 293 cell lines (flag-NLRP3 and flag-NLRP3-APEX2) were cultured in the same medium and selected by 100 µg/mL hygromycin B (InvivoGen, ant-hg-1). 1 µg/mL tetracycline was added to induce the expression of flag-NLRP3 or flag-NLRP3-APEX2.

PBMC isolation and *in vitro* culture

To isolate the mononuclear cells, leukocyte cones (around 4 mL) were diluted in 25 mL RPMI medium before laid onto 15 mL LymphoPrep without disturbing the interface between LymphoPrep and the cone blood. Subsequently, it was centrifuged at 2000 rpm (400 g) at room temperature for 20 min.

Lymphocytes, which form a thin layer of white film after the centrifuge, are transferred into a fresh 50 mL tube with a Graduated Pasteur Pipette and washed twice with RPMI medium. PBMCs were plated in 6-well plate at 1-2 × 10^7 cells/mL in 10 mL and allowed to adhere at 37°C for 4 hrs. Non-adherent cells were removed thorough washing with RPMI-1640.

hiPSC culture and differentiation to microglia

The KOLF2.1S hiPSC line used for this study was obtained with informed consent from all subjects, donors and/or their legal guardian(s) and with the approval of all the relevant institutions. All methods and experimental protocols were carried out in accordance with University of Oxford guidelines and regulations. hiPSC lines were generated previously as part of the HipSci project (REC Reference 09/H0304/77, covered under HMDMC 14/013 and REC reference 14/LO/0345). KOLF2.1S was generated from the parental KOLF2-C1 line (https://hpscreg.eu/cell-line/WTSIi018-B-1) itself a clonal isolate of KOLF2 (https://hpscreg.eu/cell-line/WTSIi018-B) by correction of a heterozygous loss of function mutation ARID2 using CRISPR based homology repair by the Cellular Operations Gene Editing team at the Wellcome Sanger Institute (Bruntraeger et al., 2019). hiPSC were cultured in OxE8 medium with daily media changes (Vaughan-Jackson et al., 2021) on Geltrex (Gibco A1413302) coated tissue culture dishes and passaged with 0.5mM EDTA. hiPSC were differentiated into microglial precursors through the formation of embryoid bodies (EB) (van Wilgenburg et al., 2013) by seeding 4 x 10^6 hiPSC per well of an Aggrewell 800 plate (STEMCELL Technologies, 34815) in EB medium (OxE8 supplemented with 50 ng/mL BMP4 (PeproTech PHC9534), 50 ng/mL VEGF (PeproTech PHC9394), and 20 ng/mL SCF (Miltenyi Biotec 130-096-695)). After differentiating in EB media for 6 days (with daily 50% media changes), 150 EB were transferred to T175 coated with 0.1% Gelatin containing differentiation media (X-VIVO 15 (Lonza BE02-060F), 1x GlutaMAX (Lifetech 25050-061), 50μM β-mercaptoethanol (LifeTech 31350-010), 100 ng/mL M-CSF (Gibco, PHC9501), 25 ng/mL IL-3 (Gibco, PHC0033). After 4-5 weeks of differentiation, microglial precursors shed into the media are harvested weekly by collection of supernatant and pelleting at 400 x g for 5 minutes. Microglial precursors are then resuspended in the final microglia maturation media ITMG (ADMEM/F12 (LifeTech, 12634-010), 1x GlutaMAX (Lifetech, 25050-061), 100 ng/mL IL-34 (PeproTech, 200-34-500), 50 ng/mL TGFβ-1 (PeproTech, 100-21C-250), 25 ng/mL M-CSF (Gibco, PHC0033), 10 ng/mL GM-CSF (Gibco, PHC2013), as previously described (Washer et al., 2022), and differentiated to hiPSC-microglia for 14 days, with 50% media changes every 3 to 4 days. All differentiation steps are carried out at 37°C, 5% CO2.

Inflammasome activation assay

THP-1 cells were plated in a concentration of 1x10^6 cells/mL and incubated with 0.5 μM PMA for 4 h. After differentiation, PMA-containing media was replaced with fresh media. Experiments were carried out on the following day. Differentiated THP-1 cells were primed with LPS (1 μg/mL) for 4 h, followed

by the treatment of PBS (mock) or 5 μM nigericin for 45 min. After stimulation, supernatants and cell lysates were collected separately, or combined together for immunoblotting analysis.

ASC speck oligomer crosslinking

PMA-differentiated THP-1 cells were primed with LPS (1 μg/mL) for 4 h, and then stimulated with nigericin (10μM) for 45 min. Cells were lysed on ice in Buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 320 mM sucrose, and protease inhibitor cocktail), and homogenized using a 21-gauge needle. The lysates were centrifuged at 1,800 rpm (300 g) for 8 min at 4 °C. Supernatants were transferred to a new tube and diluted with 1 volume of CHAPS buffer (20 mM HEPES-KOH, pH 7.5, 5 mM MgCl2, 0.5 mM EGTA, 0.1% CHAPS, and protease inhibitor cocktail). The diluted lysates were then centrifuged at 5,000 rpm (2,400 g) provide g for 8 min to obtain the crude inflammasome-containing pellets. The pellets were washed with ice cold PBS twice, resuspended in CHAPS buffer, and then cross-linked at 37 °C for 20 min by adding 2mM DSS (disuccinimidyl suberate, dissolved in DMSO). The reaction was quenched by adding SDS sample buffer and followed by Western blotting analysis.

Enzyme-linked immunosorbent assay (ELISA)

Human IL-1β (DY201, R&D Systems) and IL-6 (DY206, R&D Systems) production in the supernatants were detected by ELISA kit according to manufacturer's protocol. Plates were coated with diluted capture antibody in sterile PBS at room temperature overnight. On the following day, plates were washed three times with 200 μL/well wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) before being blocked with 200 μL/well reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 μm filtered) at room temperature for a minimum of 1 h. After blocking, plates were washed three times and incubated with samples or standards (in reagent diluent) for 2 h at room temperature. After three washes of plates, 100 μL/well detection antibody (diluted in reagent diluent) was added to the wells and incubated for 2 h at room temperature. Plates were then washed three times and incubated with 100 μL/well Streptavidin-HRP for 20 min at room temperature, followed by another three washes and incubation with 100 μL/well substrate solution (1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine)) at room temperature. 20 min after the reaction, 50 μL/well stop solution (2 N H2SO4, DY994, R&D Systems) was added and mixed thoroughly to quench the reaction. The optical density of each well was determined at the wavelength of 450nm, and the relative concentration was calculated based on the standard curve, generated through a 3 parameter logistic curve fit using the data from standard samples.

TCA/DOC protein precipitation

Trichloroacetic acid (TCA)- deoxycholate (DOC) precipitation was commonly used to concentrate the proteins in supernatants and this method was adapted from André et al. Briefly, 8.5 μL of 2% Na-

deoxycholate was added to 1 mL sample with a final concentration of 125 μg/mL. This mixture was incubated at room temperature for 15 min and 333 μL of 24% TCA (final concentration 6%) was added to the samples. Proteins were precipitated and centrifuged at 12,000 g for 30 min at 4°C. Supernatant was carefully removed and the pellets were washed once with acetone to remove excessive TCA before another centrifuge at 12,000 g for 5 min. After removing the supernatants, the resulting pellets were solubilised in loading buffer and subjected to immunoblotting analysis.

Cytotoxicity and cell viability assay

Relevant cells were treated as indicated, and the cytotoxicity was detected using CyQUANT™ LDH Cytotoxicity Assay (Invitrogen) according to manufacturer's instruction. Briefly, 50 μL of each sample supernatant was transferred to 96-well flat-bottom plate in triplicate wells, and then, 50 μL of premixed reaction buffer was added to each well. The plate was incubated for 30 min at room temperature before addition of 50 μL/well stop solution. The optical density of each well was measured at the wavelength of 490nm and 680nm (680 nm absorbance was used as background), and the cytotoxicity (% LDH release) was calculated according to manufacturer's protocol.

Cell viability was measured using CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS, Promega). Briefly, 20μl of CellTiter 96® AQueous One Solution Reagent was added to each well containing samples in 100 μl of culture medium. The plate was then incubated for a minimum of 1h at 37°C. The absorbance of each well was measured at the wavelength of 490nm, and the cell viability was calculated according to manufacturer's protocol.

Caspase-1 activity bioluminescent assay

Relevant cells were treated as indicated, and the caspase-1 activity in the culture medium (supernatant) was measured using Caspase-Glo® 1 Inflammasome Assay (Promega) according to manufacturer's instruction. Briefly, Caspase-Glo 1 Reagent was prepared through resuspension of Z-WEHD substrate using Caspase-Glo 1 buffer, and the addition of MG132 inhibitor to a final concentration of 60 μM. Afterwards, 50 μL of cell culture medium was transferred to the 96-well plate, followed by adding 5 μL Caspase-Glo 1 Reagent. The contents of the wells were thoroughly mixed and incubated at room temperature for at least 1 hour for the signal to be stable. Subsequently, the luminescence value was measured using multi-mode microplate reader FLUOstar Omega (BMG LABTECH) according to manufacturer's instruction.

Immunoprecipitation

Cells were washed with ice-cold PBS for two times, and then lysed in Pierce® IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, PhosSTOP phosphatase inhibitor and cOmplete™ Protease Inhibitor Cocktail). Lysates were incubated on ice for 10 min with periodic mixing, and then clarified at 13,000 g for 10 min at 4°C to remove the cell debris. Pre-cleared

lysates were incubated with anti-FLAG® M2 Magnetic Beads at room temperature for 30 min. After three times of washes with TBS, the proteins bound by the anti-flag antibody were pulled down and 3X FLAG® peptide was added for elution. Both the whole cell lysates and the pull-down samples are subjected to immunoblotting analysis.

Transient transfection

For DNA plasmids: Cells were grown in 6-well plates and Lipofectamine 3000 Transfection Reagent (L3000001, ThermoFisher UK) was used to transfect the cells following the manufacturer's instructions. The concentration of plasmids used was indicated in each experiment. For siRNA transfection experiments: Cells were grown in 6-well plates and RNAimax transfection reagent (Invitrogen, 13778-150) was used following the manufacturer's protocol. A final concentration of 10 nM of the following Silencer™ select siRNAs were used: control siRNA (4390844), si-NLRP3 (s41554) and si-UCH-L1 (s14616).

Construction of plasmids

The full list and descriptions of all primers and plasmids used in this study can be found in key resources table. For the generation of NLRP3-APEX2, NLRP3-Flag, NEBuilder HiFi DNA Assembly (NEB: E5520S) was used following manufacture protocol. Primers were designed using the NEBuilder Assembly Tool. The successful clones were confirmed using both PCR and DNA sequencing. Finally, the expression plasmids for NLRP3 domains were generated using the Q5® Site-Directed Mutagenesis Kit (NEB: E0554S) following manufacture protocols. We used as a template the GFP- NLRP3 plasmid (Addgene), we initially replace GFP with Flag. Finally, different domains were deleted to generate the successful plasmids. The successful clones were confirmed using both PCR and DNA sequencing.

Generation of stable cell line

The Flp-In T-Rex 293 stable cell lines (flag-NLRP3 and flag-NLRP3-APEX2) were generated using Lipofectamine 3000 following manufacture protocol in a 6-well plate. The cells were transfected with both the expression plasmid and the plasmid pOG44 in a ratio of 1:9. Cells were split the following day and transferred in a 100mm dish. After 8 hours, 300 μg/mL hygromycin B was added into the medium for selection.

sgRNA design, lentivirus production and transduction

Non-targeting control (NTC) and UCH-L1-targeting sgRNA sequences were designed using CRISPick tool (https://portals.broadinstitute.org/gppx/crispick/public). To clone the target sequences into the lentiCRISPRv2 backbone (one vector system), two oligos containing the target sequences were synthesized (Sigma) and inserted into the digested lentiviral construct. sgRNA sequences are as followed: UCH-L1-sgRNA#1: 5'-ACTTCATGAAGCAGACCATT-3'; UCH-L1-sgRNA#2: 5'-

AATCGGACTTATTCACGCAG-3'; UCH-L1-sgRNA#3: 5'- AAGACAAACTGGGATTTGGT-3'. HEK293T cell lines were seeded at 40% confluency and transfected with pMD2G, psPAX2 and the above lentiCRISPRv2 plasmids using lipofectamine 2000 following manufacturer's instructions. After incubation for two days, supernatants were collected and filtered by 0.45μm filter, followed by ultracentrifuge to obtain concentrated viral particles.

Biotin-phenol proximity labelling and biotinylated proteins capture

This method was adapted from that previously reported by Stephanie et al. 5×10^6 Flp-In T-Rex HEK293 stable cells were induced with 1 μg/mL tetracycline overnight. The next day cells were incubated with medium containing 500 μM biotin-phenol (BP) for 30 min at 37 °C under 5% CO2. Afterwards, a final concentration of 1 mM H_2O_2 was added and incubated for 40 seconds. The reaction was then quenched by the addition of quenching buffer (10mM sodium ascorbate, 10mM sodium azide, 5mM Trolox in DPBS). Subsequently, cells were washed with quenching buffer for three times, PBS for two times and then lysed with RIPA lysis buffer containing 1 mM PMSF, 5 mM Trolox, 10 mM sodium azide, 10 mM sodium ascorbate, PhosSTOP phosphatase inhibitor and cOmplete™ Protease Inhibitor Cocktail. Samples were then briefly sonicated and centrifuged at 10,000 g for 10 min at 4°C to remove cell debris. For streptavidin pull-down experiments, Dynabeads MyOne Streptavidin T1 was prepared and washed with RIPA lysis buffer twice. The cleared lysate was applied to the magnetic beads and at RT for 1 hour. Afterwards, the beads were washed for two times with RIPA lysis buffer, once with 1M KCL, 0.1 M Na_2CO_3 and 2 M Urea (in 10 mM Tris-HCL, pH 8) and finally twice with RIPA buffer again. For western blot analysis, biotinylated proteins were eluted using 60 μL of 3×loading buffer supplemented with 2 mM biotin and 20 mM DTT by boiling (98°C) for 10 min. For Mass spectrometry analysis, an on-beads digestion protocol was used as described below. Samples were denatured in 8 M Urea (in 100 mM triethylammonium bicarbonate buffer (TEAB)) and incubated at room temperature for 30 min. Afterwards, the samples were reduced with 10mM tris(2-carboxyethyl)phosphine (TCEP) for at room temperature 30 min. Samples were alkylated by the addition of iodoacetamide (final concentration 50 mM) and incubated at room temperature for 30 min. The reaction was quenched by adding DTT (final concentration 20 mM), and the urea concentration were diluted down to 1.5 M by adding TEAB. Biotinylated proteins were digested through the incubation with trypsin (1 μg trypsin per 20 μl streptavidin beads) overnight at 37 °C. Following the trypsinization, samples were desalted using the C18 solid phase cartridge (Sep-Pak, Waters) according to the manufacturer's protocol. Purified peptide eluates were dried down in the speed-vac, re-suspended in buffer A (98 % MilliQ-H20, 2 % CH_3CN and 0.1 % trifluoroacetic acid - TFA) and stored at -20 °C until further analysis.

Affinity-purification mass spectrometry (AP-MS)

1×10^7 HEK293 FLAG-NLRP3 Flp-In T-Rex stable cells were induced with 1 μg/mL tetracycline overnight. The next day cells were incubated with medium with Nigericin (10μM) or DMSO for 90 min

at 37 °C under 5% CO_2. Cells were washed with ice-cold PBS for five times, and then lysed in Pierce IP lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 5% glycerol, PhosSTOP phosphatase inhibitor and cOmplete™ Protease Inhibitor Cocktail). Lysates were incubated on ice for 10 min with periodic mixing, and then clarified at 13,000 g for 10 min at 4°C to remove the cell debris. Pre-cleared lysates were incubated with anti-FLAG M2 Magnetic Beads at 4ºC overnight. The next day beads were washed 3 times with TBS. Samples were eluted were eluted using 100 µL of 3×loading buffer supplemented 20 mM DTT by boiling (98°C) for 10 min.

Mass spectrometry analysis

An Orbitrap Fusion Lumos Tribid mass spectrometer (Thermo Fisher Scientific) coupled to an Ultimate 3000 UHPLC (Thermo Fischer Scientific) platform was used to analyse the purified tryptic peptides by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Davis et al., 2017). In brief, ten per cent of tryptic peptides (~ 200 ng) were loaded onto a trap column (PepMapC18; 300µm x 5mm, 5µm particle size, Thermo Fischer) and separated on a 50 cm EasySpray column (ES803, Thermo Fischer) using a 60 minute linear gradient ranging from 2 % to 35% buffer B (A: 5% DMSO, 0.1% formic acid; B: 5% DMSO, 0.1% formic acid in acetonitrile) at a flow rate of ~250 nL/min. Eluted peptides were then subjected to electrospray ionisation (ESI) and directed into an Orbitrap Fusion Lumos Tribrid mass spectrometer (instrument control software v3.3). Data were acquired in data-dependent mode (DDA), with the advance peak detection (APD) feature enabled. Survey scans were acquired in the Orbitrap at 120 k resolution over a m/z range 400 -1500, AGC target of 4e5 and S-lens RF of 30. Fragment ion spectra (MS/MS) were obtained in the Ion trap (rapid scan mode) with a Quad isolation window of 1.6, 40% AGC target and a maximum injection time of 35 ms, with HCD activation and 28% collision energy. Raw MS files were analysed using MaxQuant software (v1.6.10.43) using label free quantitation (LFQ) as described previously (Olie et al., 2023). In brief, raw MS files were searched against the UniProtKB human sequence database (UP000005640, 79,038 human sequence entries). The following parameters were set to perform label free quantification: Modifications were set on Carbamidomethyl (C) as fixed and Oxidation (M) and Deamidation (NQ) as variable and a maximum of two missed cleavages were allowed. Match between runs function was used.

RNA extraction and real time reverse transcription (RT)-PCR

Total RNA was isolated with the TRIzol reagent (SigmaAldrich) according to the manufacturer's instructions. The nucleic acid concentration was measured with the NanoDrop2000 (NanoDrop Technologies, Inc.). Complementary DNA (cDNA) was synthesized from 1.5 µg of total RNA with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real-time polymerase chain reaction (RT-qPCR) was performed using the LightCycler 480 SYBR Green I Master

kit and LightCycler 480 PCR System (Roche, Switzerland) according to the manufacturer's instructions. The Delta-Ct method was used to calculate the relative RNA expression levels.

Pathway enrichment analysis

Filtered proximity proteomes (SAINT probability > 0.8, FC-A ≥ 1) either in DMSO control or nigericin activated samples were imported into the STRING database search portal (version 11.5, Szklarczyk et al., 2021; von Mering et al., 2003), and the top 20 retrieved terms were plotted with both strength (Log10 [observed / expected]) and false discovery rate (FDR) (interaction score ≥ 0.7). Terms were clustered through the similarity of enrichment strength patterns across the four groups and the results were further visualized using clusterProfiler 4.0 (Wu et al., 2021; Yu et al., 2012).

PPIs network construction

Differentially enriched proteins under unstimulated or stimulated condition that passed the filtering criteria (log2[FC]≥0.5 or ≤-0.5, FDR≤ 0.05) were separately uploaded to the STRING portal, and physical subnetwork type was chosen to extract the physical (direct) protein-protein associations from the databases. Active interaction sources, including text mining, experiments, and databases, and an interaction score > 0.7 (high confidence interaction) were applied to construct the PPI networks. Markov clustering algorithm (MCL) was used to group the interaction subnetworks with the inflation value of 3.0, resulting in 13 clusters for the stimulated condition and 18 clusters for the unstimulated condition.

SAINT probabilistic scoring for NLRP3 APEX2 PL-MS and AP-MS data

The computational tool, SAINTexpress (Teo et al., 2014)- an updated version of the "Significance Analysis of INTeractome (SAINT)," was utilised to allocate confidence scores to the proximity labelling and AP-MS data. This analysis was conducted via the online Contaminant Repository for Affinity Purification platform at www.crapome.org (Mellacheruvu et al., 2013).

Statistics and reproducibility

Data are presented as mean ± standard error of the mean (SEM). Two-tailed, unpaired student's t test was used for statistical analysis in GraphPad Prism 9. Unless stated, otherwise all quantitative experiments were performed in triplicates and average with SEM reported.

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Deutsch et al., 2023; Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD045862.

Acknowledgements

Z.L., A.D., I.V., G.L., X.Z., R.F., T.D. and B.M.K. were supported by the Chinese Academy of Medical Sciences (CAMS) Innovation Fund for Medical Science (CIFMS), China (grant number: 2018-I2M-2-002) awarded to B.M.K., R.F. and T.D. B.M.K. was supported by Pfizer. E.D. was supported by Alzheimer's Research UK Grants ARUK2015DDI-OX. E.J was funded by Wellcome Trust (grant # 224040/Z/21/Z). Imaging was performed using the Oxford-Zeiss Centre of Excellent in Biomedical Imaging and the Kennedy Trust for Rheumatology Research (grant # 202117 and 202103, respectively). E.W.T. was supported by a CRUK Programme Award (DRCNPG-Nov21\100001) with support from the Engineering & Physical Sciences Research Council. We would also like to thank members of the Discovery Proteomics Facility, the Kessler, Oxford Drug Discovery Institute groups for constructive discussions and Raphael Heilig for his expert help with mass spectrometry.

Author contributions

B.M.K., E.D. and Z.L. designed the research and wrote the manuscript. Z.L. and A.D. performed the NLRP3-APEX2 proximity labelling experiments and analysed the mass spectrometry data. Z.L. and A.D. performed the DUB siRNA library screen experiment. I.V. operated mass spectrometer for LC-MS/MS acquisition of the samples and contributed to data processing. E.J. performed confocal imaging and analysis. S.J.W. and A.D. contributed to the culture of hiPSC and differentiated them into microglial cells. E.W.T. and F.C. contributed to UCH-L1 inhibitor provision and study design. Z.L., A.D., I.V., E.J., F.H.L., S.J.W., G.L., G.Y., H.L., F.C., X.Z., R.F., T.D., E.W.T., E.D. and B.M.K discussed the results, commented, and provided critical reviews on the manuscript.

Competing interests

E.W.T. is a founder and shareholder in Myricx Pharma Ltd, and receives consultancy or research funding from Kura Oncology, Pfizer Ltd, Samsara Therapeutics, Myricx Pharma Ltd, MSD, Exscientia and Daiichi Sankyo. The other authors declare no competing interests.

Figure S1

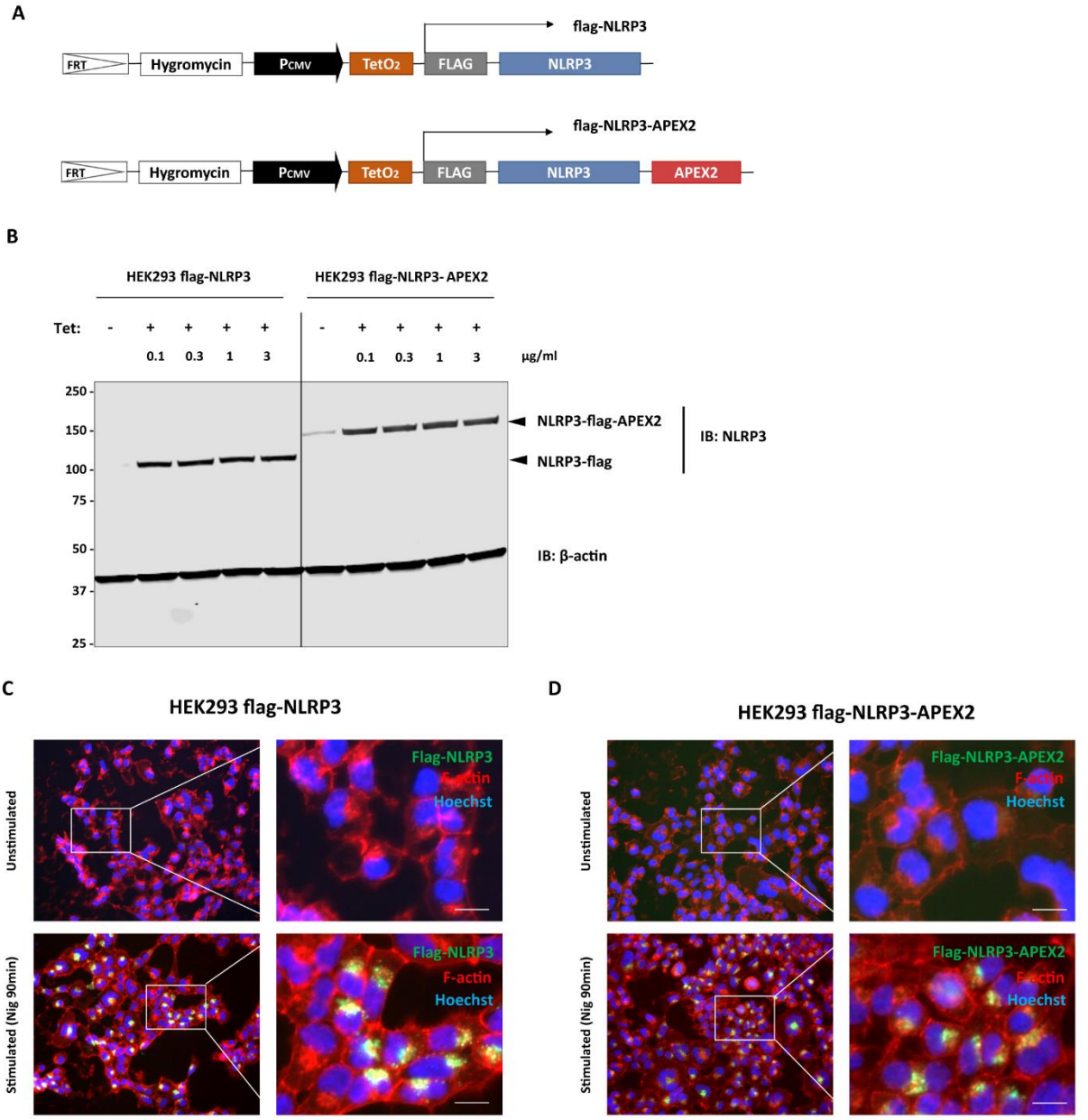


Figure S1 | Construction of flag-NLRP3 and flag-NLRP3-APEX2 HEK293 stable cell line

(A) Schematic of Flag-NLRP3 and Flag-NLRP3-APEX2 constructs. (B) Both the flag-NLRP3 and flag-NLRP3-APEX2 Flp-In T-Rex HEK293 stable cell lines were treated with tetracycline at indicated concentrations (0.1-3 μg/mL). 24 hours after induction, cell lysates were collected for immunoblotting analysis. (C-D) Immunofluorescence of flag-NLRP3 (C) and flag-NLRP3-APEX2 (D) in Flp-In T-Rex HEK293 stable cell lines. Cells were either treated with DMSO or Nigericin (10μM) for 90 min. Scale bars, 10 μm.

Figure S2

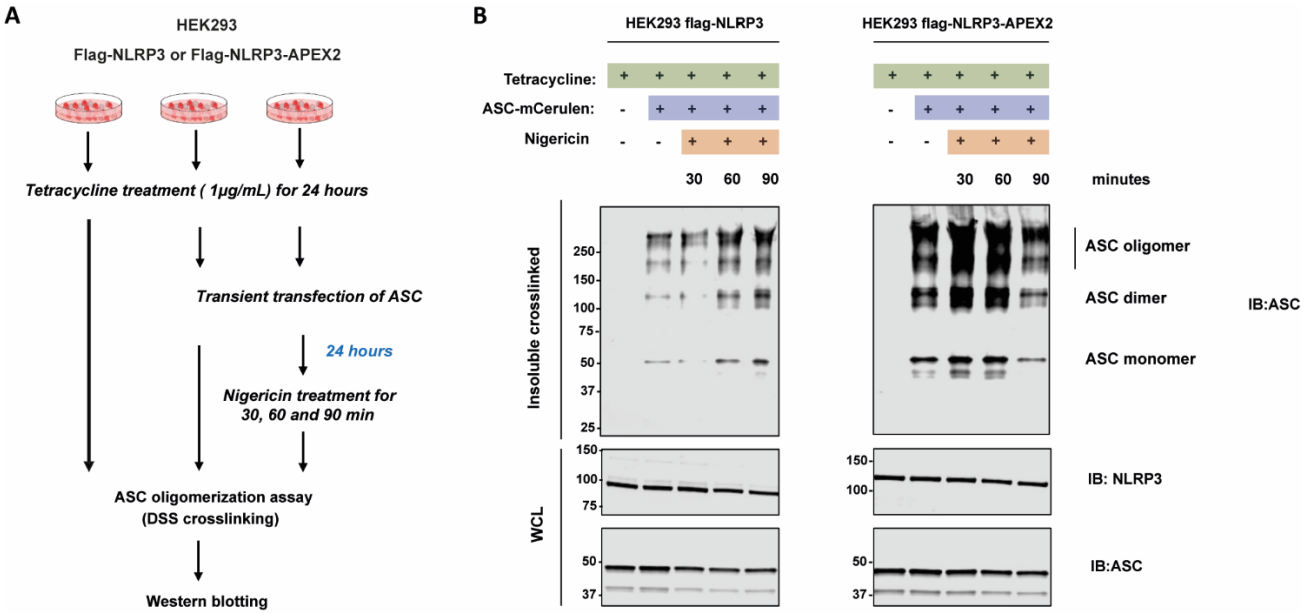


Figure S2 | Reconstituted NLRP3 induces ASC oligomerization in HEK293 cell lines

(A) Schematic of the ASC oligomerization assay in Flp-In T-Rex HEK293 stable cell lines. (B) Immunoblot of DSS-crosslinked ASC oligomers in the lysates from flag-NLRP3 and flag-NLRP3-APEX2 HEK293 stable cell lines, treated with tetracycline overnight, transfected with ASC mCerulean, and followed by the treatment with nigericin (10µM) for the indicated time.

Figure S3

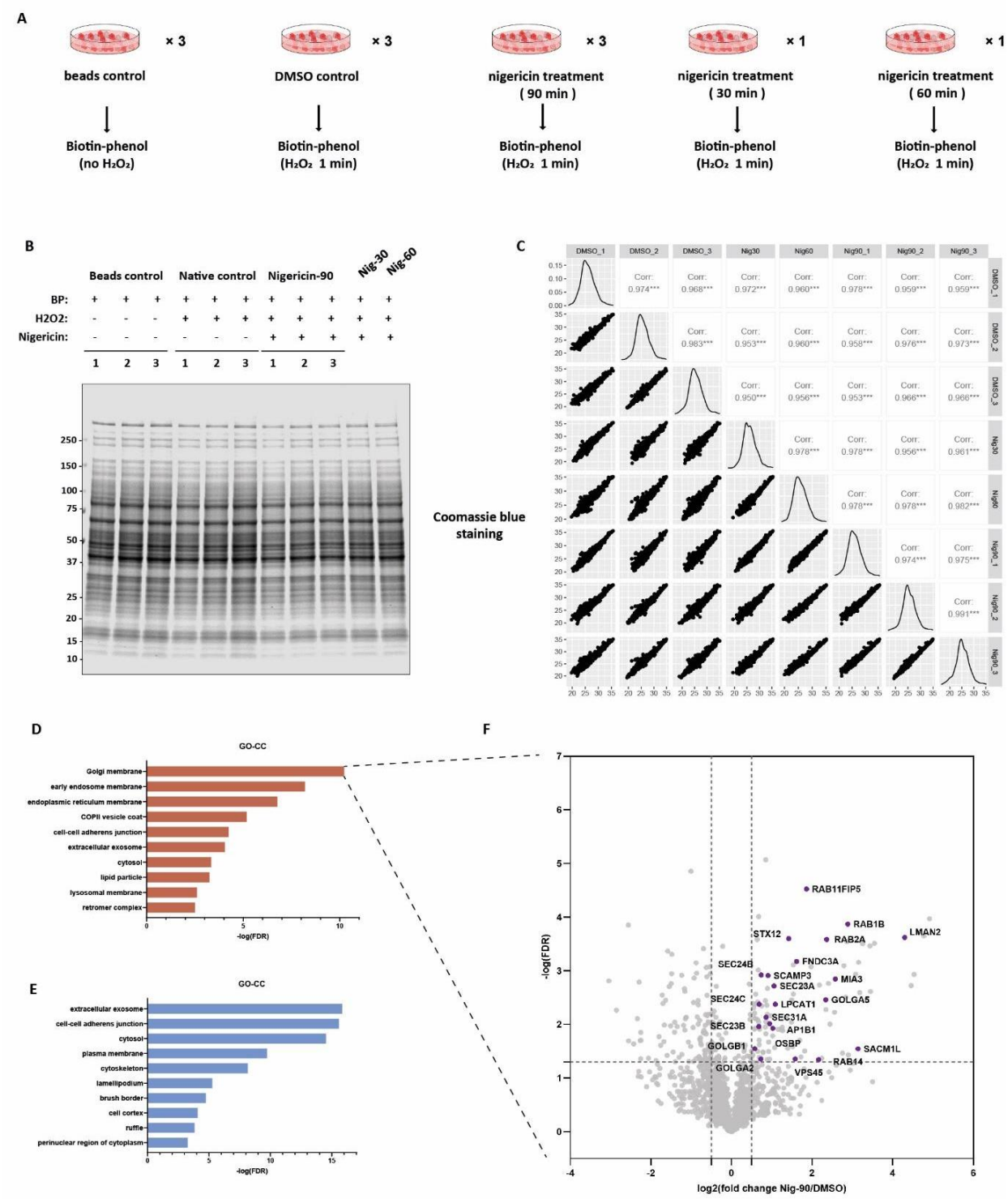


Figure S3 | Tracing NLRP3 translocation using NLRP3-APEX2 proximity labelling

(A) Scheme of the experimental design and procedures of NLRP3-APEX2 proximity labelling. (B) Coomassie blue staining of whole-cell lysates from HEK293 Flp-In T-Rex NLRP3-APEX2 cells, treated as indicated with tetracycline, biotin-phenol, H_2O_2, and nigericin. (C) Pearson correlation matrix heatmap of the biotinylated proteome profiling. This analysis confirms high reproducibility in protein

relative abundance measurements. (D-F) Bar graph showing the top 10 gene ontology-cellular component (GO-CC) terms of the proteins enriched in nigericin-stimulated state (nigericin treatment for 90 min) (D) and unstimulated state (DMSO treatment for 90 min) (E) respectively. (F) The 24 protein that belong to the Golgi membranes term are shown and coloured as purple in the volcano plotting.

Figure S4

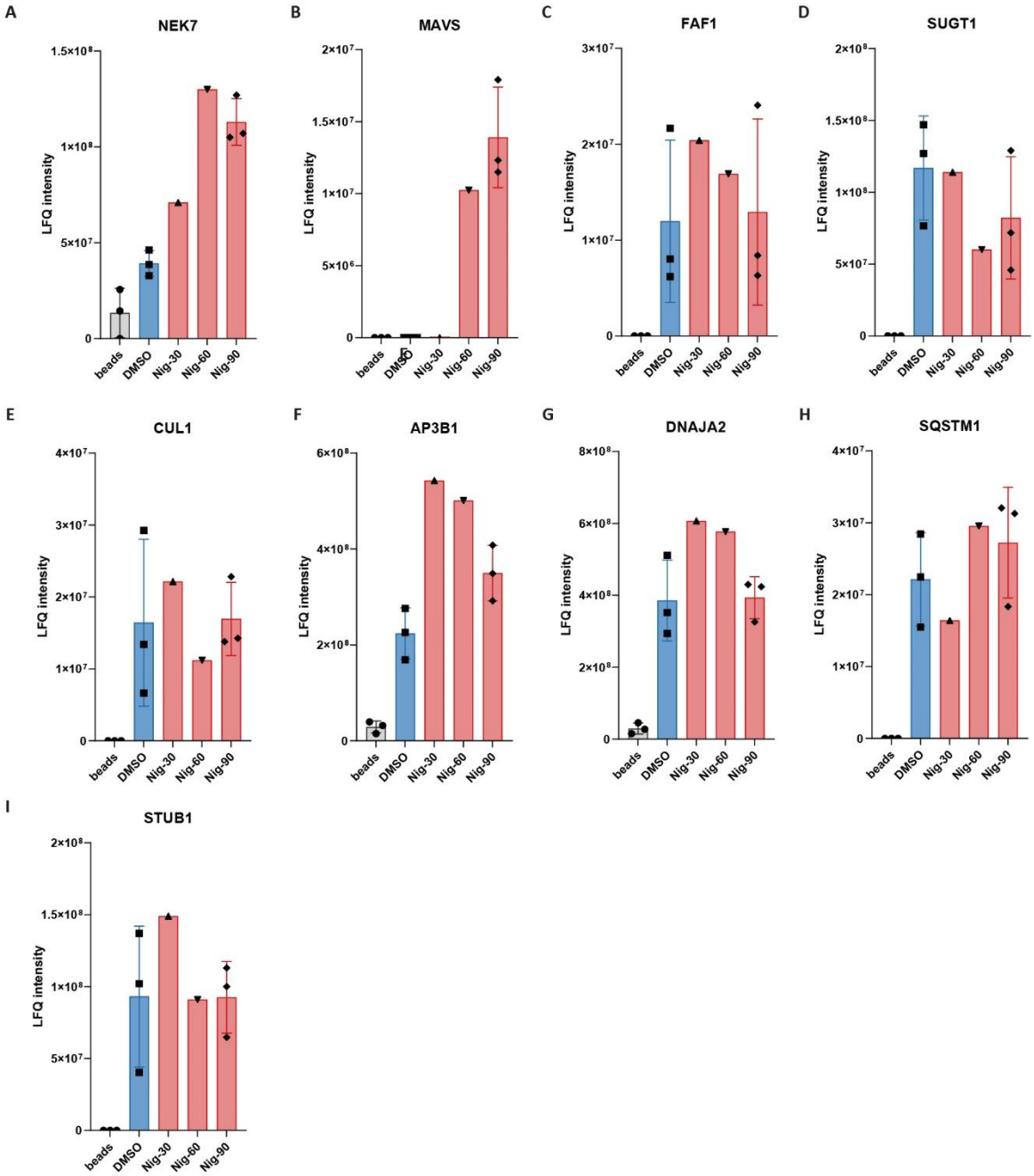


Figure S4 | APEX2 proximity labelling identifies known NLRP3-interacting proteins during NLRP3 activation downstream of potassium efflux

(A-I) The raw LFQ Intensity values of the APEX2 NLRP3 proximity labelling from all the known NLRP3-interacting proteins identified in our experiment. An average value of 3 independent experiments is presented for DMSO and Nig-90 whereas for Nig-30 and Nig-60 just a single experiment. Error bars show the standard deviation of the mean.

Figure S5

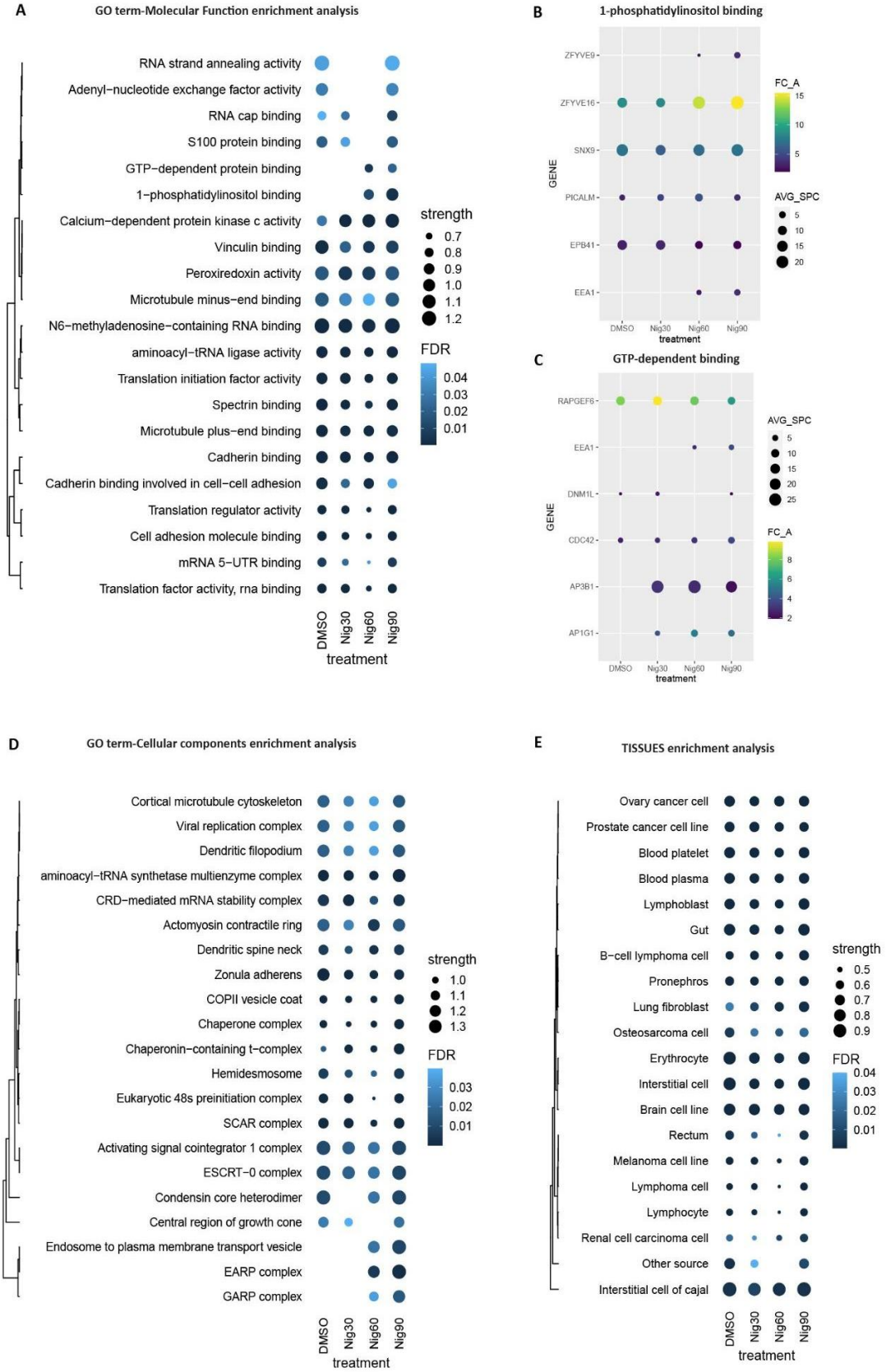


Figure S5 | Functional analysis of the NLRP3 proximal proteins during inflammasome activation

(A, D, E) Dot plots displaying the GOBP (A), GOCC (D) and TISSUE (E) enrichment analysis of high confidence NLRP3 proximal proteins upon nigericin stimulation for different timepoints as indicated. (B, C) Dot plots showing the average spectral counts (AVG_SPC) and fold change (FC_A) of NLRP3 proximal proteins enriched in the GOMF term 1-phosphatidylinostol binding (B) and GTP-dependent binding (C) respectively.

Figure S6

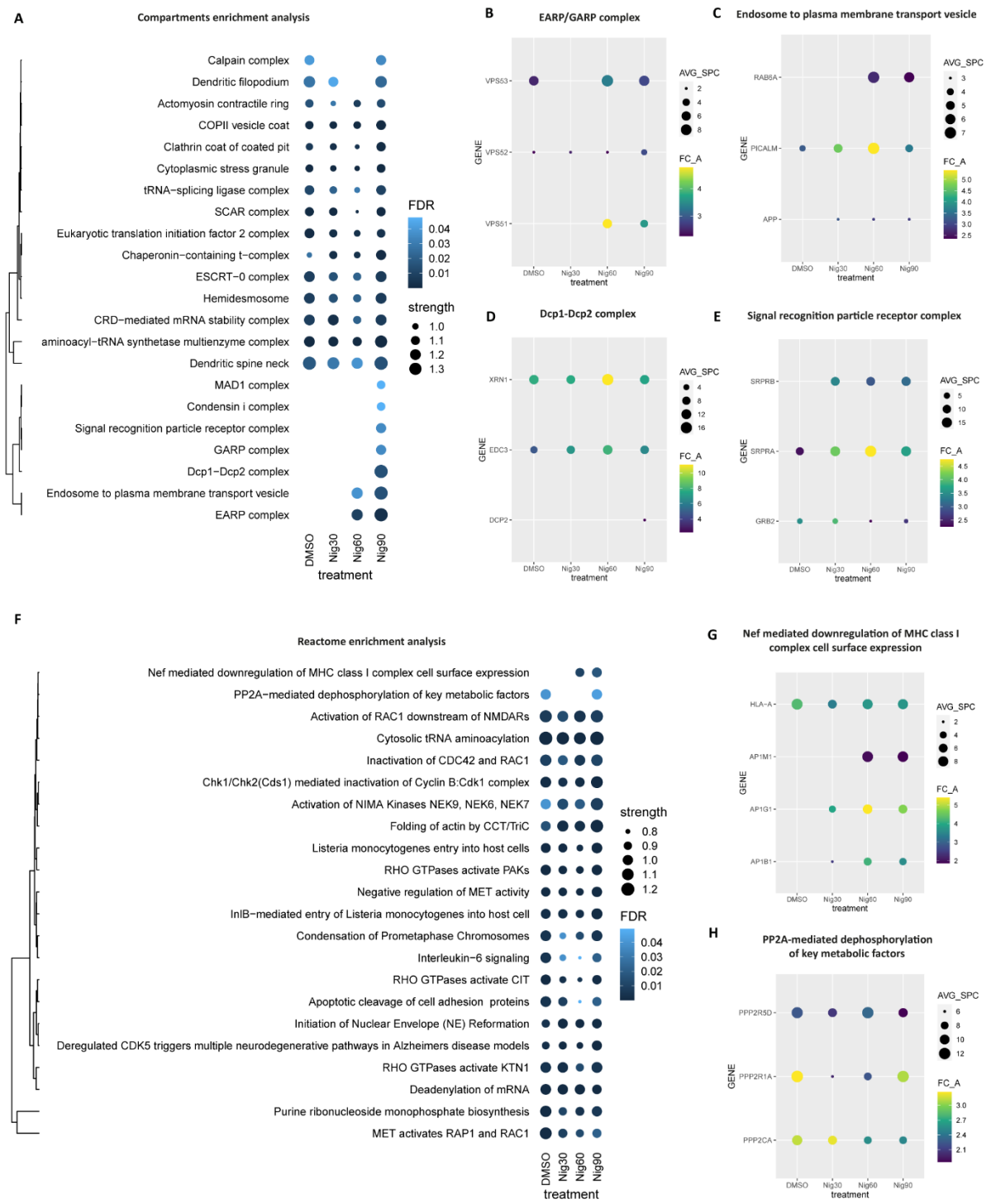


Figure S6 | Functional analysis of the NLRP3 proximal proteins during inflammasome activation (A, D) Dot plots displaying the Compartments (A) and Reactome (F) enrichment analysis of high confidence NLRP3 proximal proteins upon nigericin stimulation for different timepoints. (B-E, G-H) Dot plots showing the average spectral counts (AVG_SPC) and fold change (FC_A) of NLRP3 proximal proteins enriched in the term EARP/GARP complex (B), Endosome to plasma membrane transport vesicle (C), Dcp1-Dcp2 complex (D) and Signal recognition particle receptor complex (E),

Nef mediated downregulation of MHC class I complex cell surface expression (G), PP2A-mediated dephosphorylation of key metabolic factors (H) respectively.

Figure S7

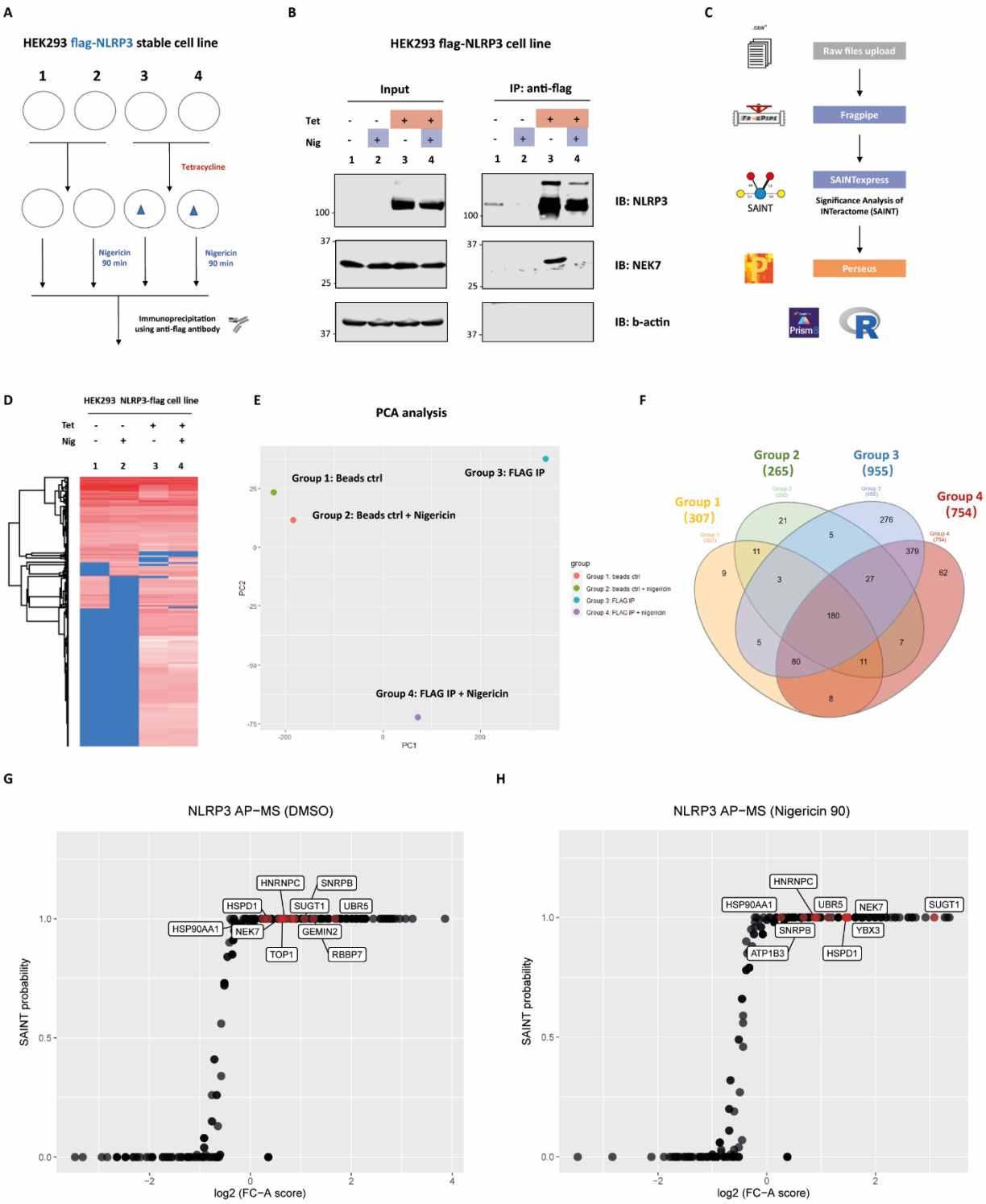


Figure S7 | NLRP3 interactome determined by AP-MS

(A) Schematic of the NLRP3 AP-MS experiment design. (B) Stable HEK293 cells expressing Flag-NLRP3 were incubated with tetracycline overnight. Subsequently, they were treated with nigericin for 90 min as specified. NLRP3-flag was then isolated using an anti-flag antibody and subjected to

immunoblotting with the indicated antibodies. (C) Diagram illustrating the data analysis workflow for AP-MS data. (D) Heatmap showing the hierarchical clusters of prey proteins in the NLRP3 AP-MS across the four groups. (E) Principal component analysis of the four samples. Each dot represents an individual sample. (F) Venn diagram illustrating the number of proteins identified from different groups as indicated in (A). (G-H) SAINT probability scoring of the proteomic data for NLRP3 AP-MS compared to the negative beads control. Proteins passing the cut-off (SAINT probability ≥ 0.8, FC-A ≥ 1) are considered as high-confidence interacting proteins for NLRP3.

Figure S8

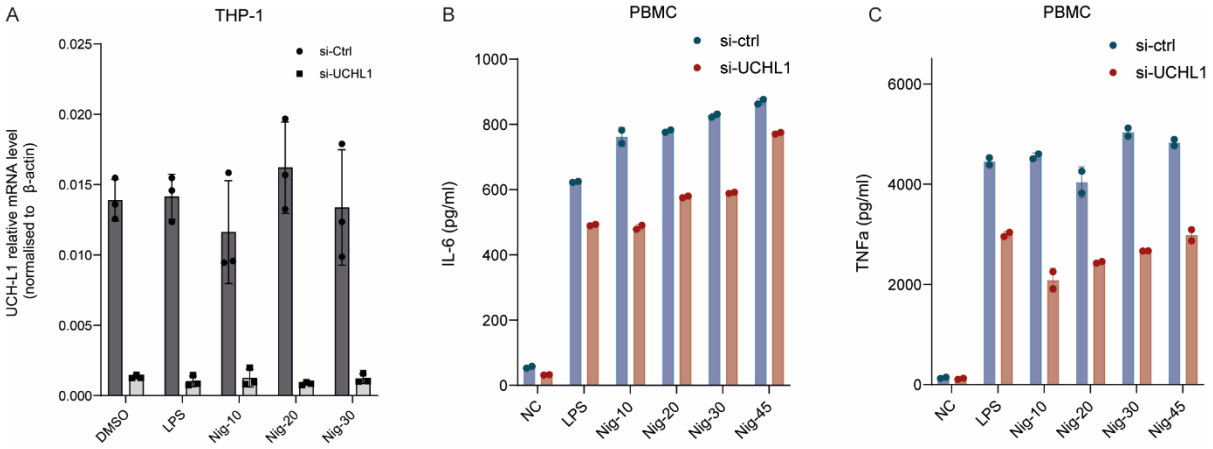


Figure S8 | UCH-L1 deficiency decreases IL-6 and TNFα expression in human PBMC

(A) RT-qPCR analysis of UCH-L1 expression in THP-1 cells after transfection with either non-targeting siRNA or UCH-L1-targeted siRNA. Following differentiation with PMA, cells were primed using LPS (1 μg/mL) for 4 hours, and then treated with nigericin (10 μM) for different timepoints as indicated. (B-C) PBMCs were transfected with non-targeting control, or UCH-L1-specific siRNA, stimulated with LPS for 4 hours and nigericin for different timepoints as indicated. Production of IL-6 (B) and TNFα (C) in the supernatants from PBMCs were measured by ELISA.

Figure S9

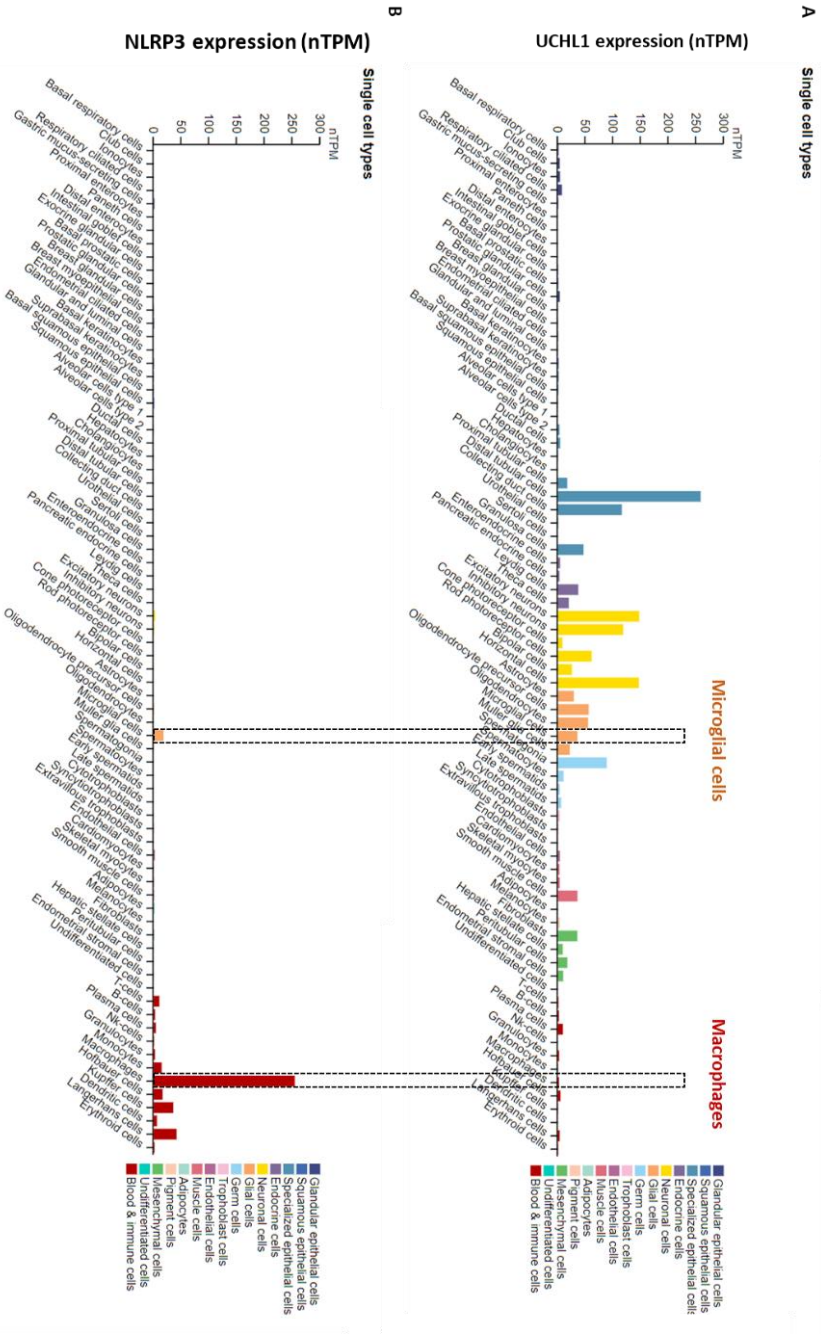


Figure S9 | RNA expression level of NLRP3 and UCH-L1 in different cell types

(A,B) A summary of normalized single cell RNA expression (nTPM) in different cell lines for UCH-L1 (A) and NLRP3 (B). Color-coding represents different cell type groups consisting of cell types with functional features in common (Data were retrieved from https://www.proteinatlas.org/)

Figure S10

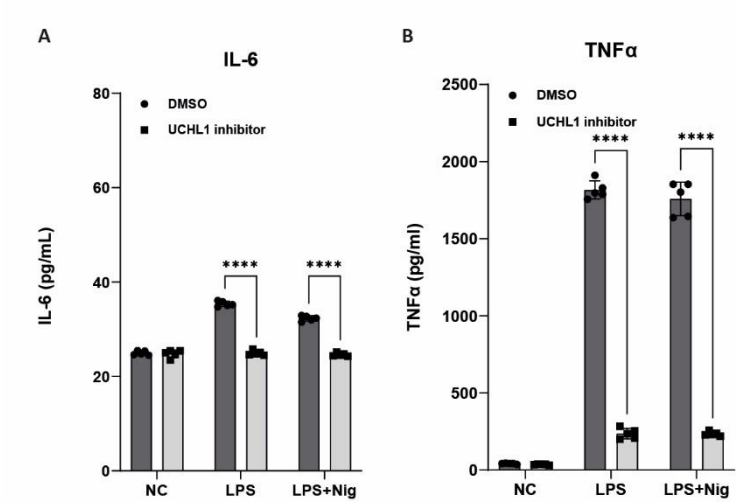


Figure S10 | UCH-L1 inhibitor diminishes IL-6 and TNFα expression in THP-1 cells

(A, B) IL-6 (A) and TNFα (B) levels determined by ELISA from THP-1 cells treated with DMSO or 0.3 μM IMP-1711-S. Cells were differentiated with PMA and primed with LPS 1 μg/mL) for 4 hours, followed by the treatment of nigericin (10 μM) for 45 minutes. The inhibitor or the DMSO were added at the same time with LPS. Error bars show the standard deviation of the mean. ****$p < 0.0001$ (unpaired two-sided t-test).

Figure S11

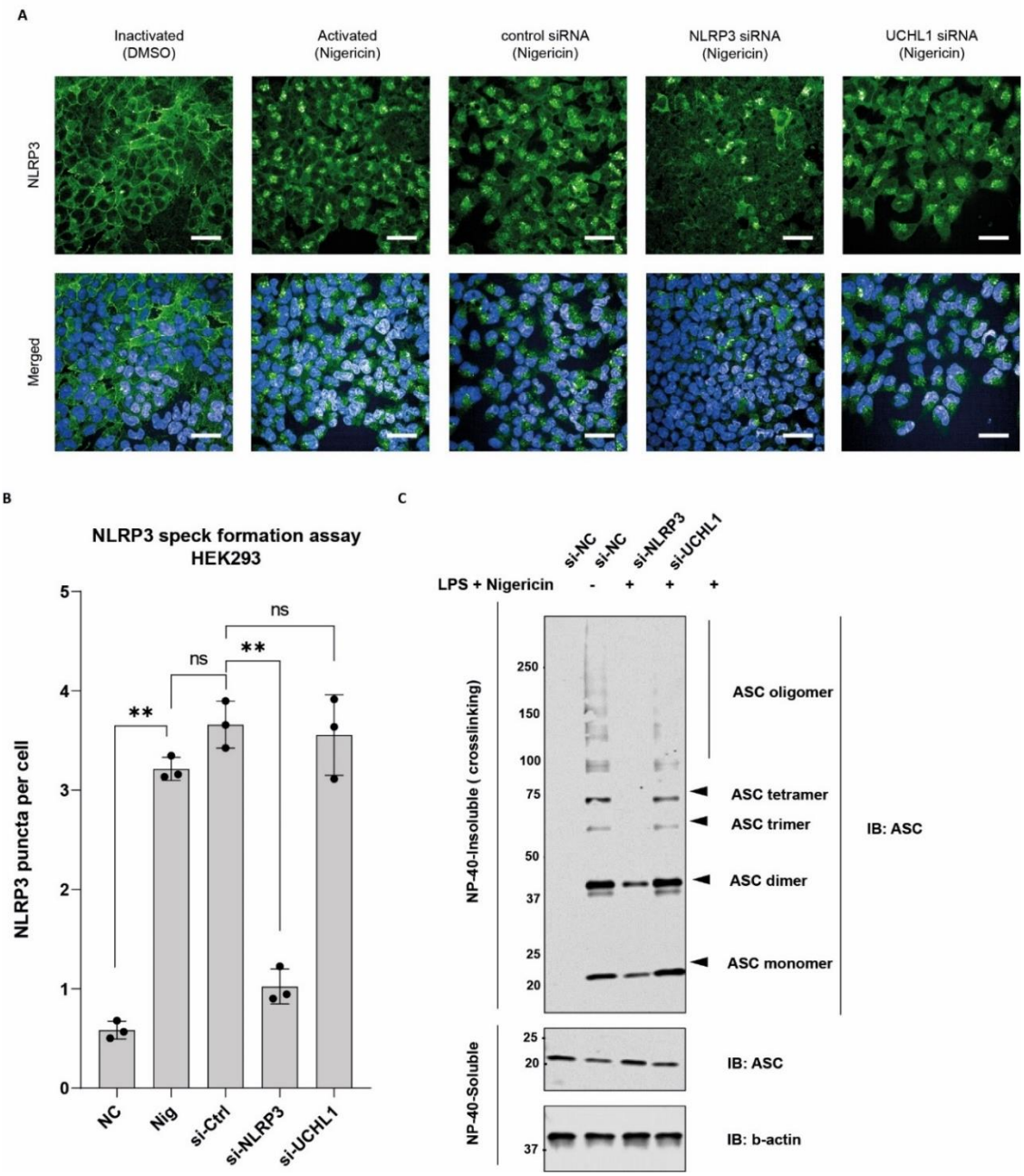


Figure S11 | UCH-L1 is dispensable for NLRP3 puncta formation but partially alters ASC oligomerization

(A, B) flag-NLRP3 HEK293 stable cells were transfected with non-targeting control siRNA, NLRP3 or UCH-L1-specific siRNA. 48 hours after transfection, cells were treated with DMSO or 10 μM nigericin for 45 minutes. Subsequently, cells were stained with anti-flag antibody to measure the formation of NLRP3 puncta. (A) Representative fluorescence imaging of NLRP3 puncta using Opera Phenix Plus High-Content Screening System. Scale bars, 50 μm. (B) Quantitative analysis of the NLRP3 puncta formation (n = 3, mean ± s.d.). (C) Immunoblot analysis of ASC after DSS crosslinking in THP-1 cells transfected with non-targeting control siRNA, NLRP3 or UCH-L1-specific siRNA.

Figure S12

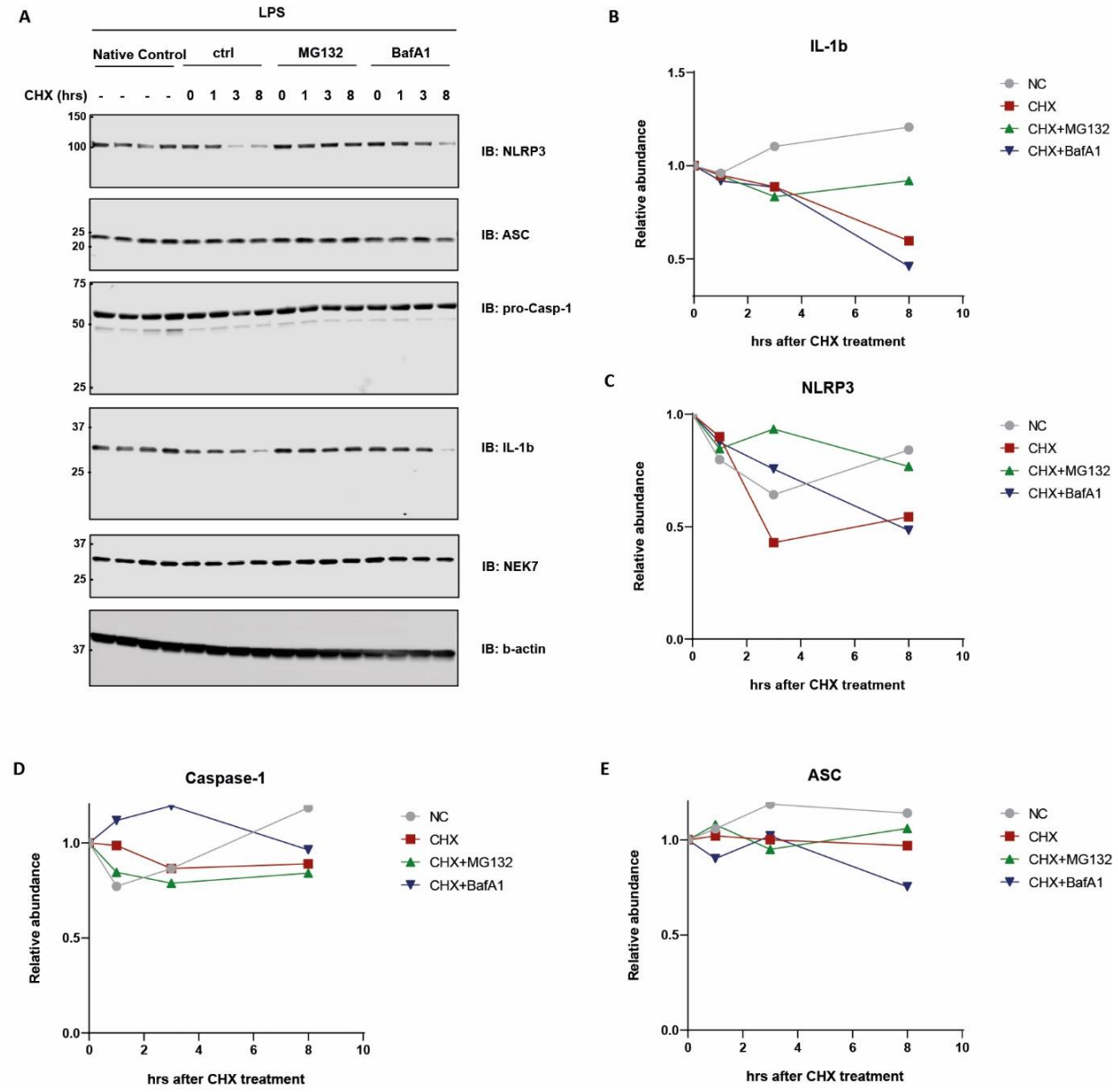


Figure S12 | Protein turnover of NLRP3 and IL-1β depend on the ubiquitin proteasome pathway

(A) PMA-differentiated THP-1 cells were primed with LPS (100ng/mL) for 4 hours and followed by the treatment of MG132 (20μM) or BafA1 (300nM) as indicated in the last 30 min alongside. Cells were then treated with cycloheximide (50μg/mL) for 1, 3 and 8 h as indicated. Cell lysates were collected and immunoblotted for the indicated antibodies. Immunoblotting from one representative experiment of three is shown. (B-E) Quantitative analysis of the western blotting results in (A).

Figure S13

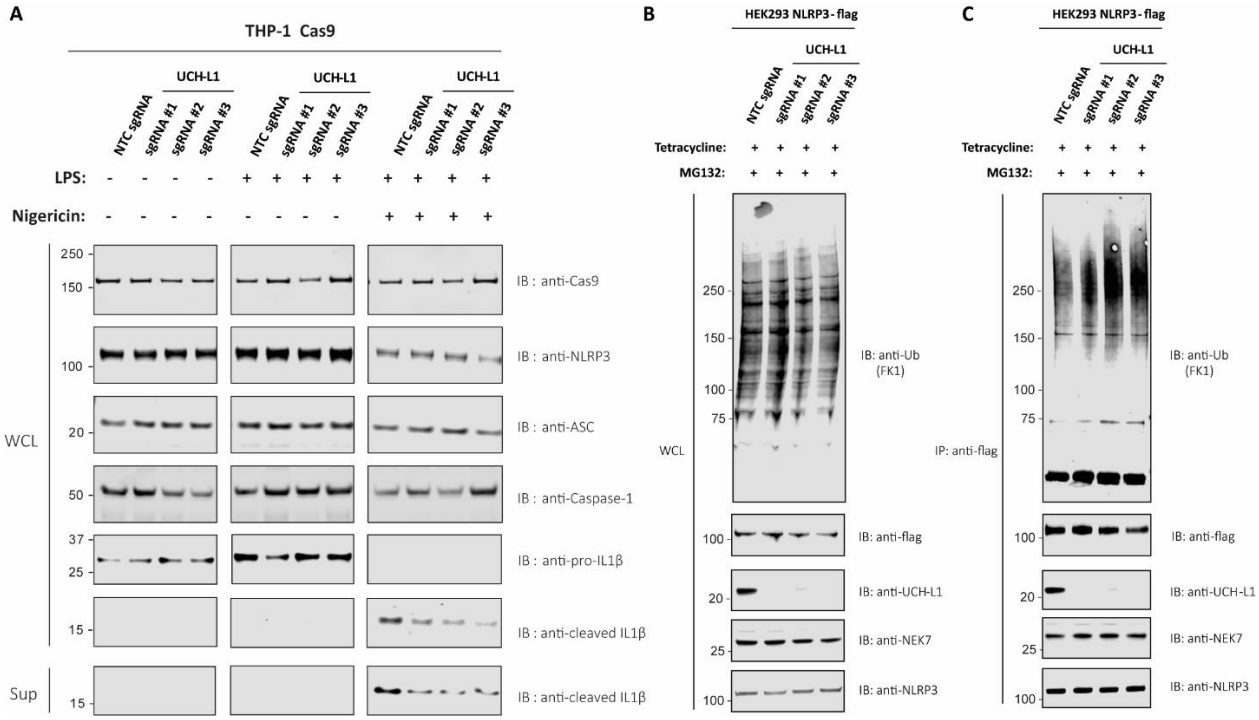


Figure S13 | UCH-L1 deficiency abrogates IL-1β production and alters NLRP3 ubiquitination

(A) THP-1 cells, transduced by lentiviruses encoding Cas9 and non-targeting control (NTC) sgRNA or sgRNA-1/2/3 targeting UCH-L1, were differentiated with PMA, primed with LPS for 4 hours and treated with nigericin for 45 minutes, as indicated. Whole cell lysates and supernatants were immunoblotted with indicated antibodies. (B-C) flag-NLRP3 HEK293 stable cell lines, transduced by lentiviruses encoding Cas9 and non-targeting control (NTC) sgRNA or sgRNA-1/2/3 targeting UCH-L1, were treated with MG132 (10 μM) for 6 hours. Whole cell lysates (A) and flag-NLRP3 immunoprecipitated in RIPA lysis buffer (B) were analysed with indicated antibodies.

Table S1 | List of known NLRP3-interacting proteins curated from literature

Interactor	Experimental System	Throughput	Organism	Reference
HIST1H1B	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
HNRNPC	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
RBBP7	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
SNRPB	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
SPAST	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
TOP1	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
LTBP4	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
SMARCA5	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
GEMIN2	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
YBX3	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
AP3B1	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
PHF14	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
URB1	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
DNAJA2	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
CBX3	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
FBXO28	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
CBX5	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
MTCH1	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
PVRL3	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
FBXL6	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
UQCRQ	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
COPS7A	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
LUC7L3	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
GPATCH4	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
FEM1A	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
LUC7L	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610

Interactor	Experimental System	Throughput	Organism	Reference
DMAP1	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
PHRF1	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
CCDC86	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
PCGF5	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
ZC3H8	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
KIAA2013	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
NLRP5	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:26496610
STUB1	Affinity Capture-MS	Low Throughput	Homo sapiens	PUBMED:25594175
MARCH7	Affinity Capture-MS	Low Throughput	Homo sapiens	PUBMED:25594175
UBR5	Affinity Capture-MS	Low Throughput	Homo sapiens	PUBMED:25594175
CUL4A	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:30945288
CUL4B	Affinity Capture-MS	High Throughput	Homo sapiens	PUBMED:30945288
VDR	Affinity Capture-MS, Affinity Capture-Western, Reconstituted Complex	High Throughput	Homo sapiens	PUBMED:31866999
NLRC4	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:15107016
SUGT1	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:17435760
HSP90AA1	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:17435760
DHX33	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:25172487
ULK1	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:26347139
MEFV	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:26347139
HDAC6	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:26471297
USP50	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:28094437
ARIH2	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:29021376
TXNIP	Affinity Capture-Western, Two-hybrid	Low Throughput	Homo sapiens	PUBMED:28326454, PUBMED:30833078, PUBMED:32323100, PUBMED:32716108
RBX1	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:30653357
FAM175B	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:30787184
BRCC3	Affinity Capture-Western	Low Throughput	Homo sapiens, Mus musculus	PUBMED:30787184, PUBMED:31866999, PUBMED:32941940

Interactor	Experimental System	Throughput	Organism	Reference
ORF8b	Affinity Capture-Western	Low Throughput	Severe acute respiratory syndrome-related coronavirus	PUBMED:31231549
SRC	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:31553908
NEK7	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:31762063, PUBMED:31866999
Ppp2ca	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:31866999
SQSTM1	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:32003754
IL1B	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:32597834
EIF3F	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:33243234
USP13	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:33243234
PSMB8	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:32782149
PARK2	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:33527027
BTRC	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:33976226
TRIM24	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:33724611
USP5	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:34486483
TRIM28	Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:34373456
LYN	Affinity Capture-Western	Low Throughput	Homo sapiens	PMID: 34699250
EIF2AK2	Affinity Capture-Western	Low Throughput	Homo sapiens	PMID: 22801494
PRKD1/2/3	Affinity Capture-Western	Low Throughput	Homo sapiens	PMID: 28716882
FBXL2	Affinity Capture-Western, Biochemical Activity	Low Throughput	Homo sapiens	PUBMED:26037928
PYCARD	Affinity Capture-Western, Co-localization	Low Throughput	Homo sapiens	PUBMED:30182366, PUBMED:32003754, PUBMED:32435622
P2RY2	Affinity Capture-Western, Co-localization	Low Throughput	Homo sapiens	PUBMED:31553908
MAVS	Affinity Capture-Western, Co-localization	Low Throughput	Homo sapiens	PMID: 23582325
NLRP11	Affinity Capture-Western, Co-localization	Low Throughput	Homo sapiens	PMID: 35624206
CBLB	Affinity Capture-Western, Reconstituted Complex	Low Throughput	Homo sapiens	PUBMED:31999304

Interactor	Experimental System	Throughput	Organism	Reference
RNF125	Affinity Capture-Western, Reconstituted Complex	Low Throughput	Homo sapiens	PUBMED:31999304
STAMBP	Biochemical Activity, Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:32003754, PUBMED:33253913
MCFD2	Co-fractionation	High Throughput	Homo sapiens	PUBMED:26344197
ARHGAP17	Co-fractionation	High Throughput	Homo sapiens	PUBMED:26344197
SH3BP1	Co-fractionation	High Throughput	Homo sapiens	PUBMED:26344197
CASP8	Co-localization	Low Throughput	Homo sapiens	PUBMED:30182366
MARK4	Co-localization	Low Throughput	Homo sapiens	PUBMED:31167564
HSPD1	Proximity Label-MS	High Throughput	Homo sapiens	PUBMED:30021884
Rnf213	Reconstituted Complex	High Throughput	Homo sapiens, Mus musculus	PUBMED:31999304
Trim21	Reconstituted Complex	High Throughput	Homo sapiens, Mus musculus	PUBMED:31999304
Trim47	Reconstituted Complex	High Throughput	Homo sapiens, Mus musculus	PUBMED:31999304
Trim14	Reconstituted Complex	High Throughput	Homo sapiens, Mus musculus	PUBMED:31999304
ORF8	Reconstituted Complex, Affinity Capture-Western	Low Throughput	Severe acute respiratory syndrome coronavirus 2	DOI:10.1101/2021.12.02.470978
TRIM31	Reconstituted Complex, Affinity Capture-Western, Biochemical Activity	Low Throughput	Homo sapiens	PUBMED:27929086
PGM1	Two-hybrid	High Throughput	Homo sapiens	PUBMED:30653357
ATP1B3	Two-hybrid	High Throughput	Homo sapiens	PUBMED:30653357
CUL1	Two-hybrid, Affinity Capture-Western, Co-localization, PCA	High Throughput	Homo sapiens	PUBMED:30653357
PXN	Two-hybrid, Affinity Capture-Western, Reconstituted Complex, Co-localization	Low Throughput	Homo sapiens	PUBMED:33243234
FAF1	Two-hybrid, Reconstituted Complex, Affinity Capture-Western	Low Throughput	Homo sapiens	PUBMED:17046979

Table S2 | List of the Golgi membrane proteins differentially captured by NLRP3-APEX2 during nigericin-induced inflammasome assembly

Protein IDs	Protein names	Gene names	Peptides	Unique peptides	Sequence coverage [%]
O14828	Secretory carrier-associated membrane protein 3	SCAMP3	11	11	42.1
O94979	Protein transport protein Sec31A	SEC31A	26	26	23
O95487	Protein transport protein Sec24B	SEC24B	23	23	22.7
P22059	Oxysterol-binding protein 1	OSBP	20	20	33
P53992	Protein transport protein Sec24C	SEC24C	25	24	31.9
P61019;Q8WUD1	Ras-related protein Rab-2A;Ras-related protein Rab-2B	RAB2A;RAB2B	6	6	37.7
P61106	Ras-related protein Rab-14	RAB14	12	12	65.6
Q00610	Clathrin heavy chain 1	CLTC	57	57	39.6
Q08379	Golgin subfamily A member 2	GOLGA2	22	22	28
Q10567	AP-1 complex subunit beta-1	AP1B1	19	5	21.6
Q12907	Vesicular integral-membrane protein VIP36	LMAN2	11	11	37.9
Q14789	Golgin subfamily B member 1	GOLGB1	106	106	45.4
Q15436	Protein transport protein Sec23A	SEC23A	20	17	35.7
Q15437	Protein transport protein Sec23B	SEC23B	24	21	37.2
Q86Y82	Syntaxin-12	STX12	8	8	37.7
Q8NF37	Lysophosphatidylcholine acyltransferase 1	LPCAT1	4	4	10.7
Q8TBA6	Golgin subfamily A member 5	GOLGA5	22	22	41.9
Q9BXF6	Rab11 family-interacting protein 5	RAB11FIP5	12	12	32.3
Q9H0U4;Q92928	Ras-related protein Rab-1B;Putative Ras-related protein Rab-1C	RAB1B;RAB1C	4	2	29.4
Q9NRW7	Vacuolar protein sorting-associated protein 45	VPS45	11	11	19.6
Q9NTJ5	Phosphatidylinositide phosphatase SAC1	SACM1L	11	11	19.3
Q9Y2H6	Fibronectin type-III domain-containing protein 3A	FNDC3A	7	7	8.3

Table S3 | Human DUB siRNA screening in THP-1 cells

LPS

Rank	Gene	Normalized IL1b	z-score
1	TNFAIP3	2.334	3.451
2	UCHL3	2.217	2.720
3	CYLD	2.103	2.013
4	USP8	2.016	1.470
5	JOSD2	2.010	1.432
6	USP38	1.996	1.343
7	USP18	1.992	1.320
8	JOSD1	1.992	1.319
9	USP40	1.983	1.261
10	OTUD6B	1.977	1.228
11	ZRANB1	1.976	1.221
12	OTUD7A	1.967	1.162
13	OTUD7B	1.956	1.098
14	USP33	1.951	1.066
15	USP46	1.938	0.984
16	USP43	1.923	0.892
17	STAMBPL1	1.914	0.838
18	OTUB2	1.902	0.759
19	USPL1	1.900	0.746
20	OTUD5	1.898	0.736
21	USP21	1.895	0.715
22	USP48	1.893	0.704
23	USP3	1.870	0.563
24	STAMBP	1.867	0.542
25	USP45	1.863	0.518
26	USP27X	1.863	0.518
27	USP11	1.860	0.501
28	USP13	1.856	0.472
29	COPS5	1.854	0.464
30	USP6	1.848	0.422
31	MYSM1	1.845	0.403
32	USP50	1.838	0.361
33	PAN2	1.832	0.326
34	SENP2	1.832	0.323
35	USP5	1.831	0.317
36	USP24	1.831	0.315
37	PRPF8	1.821	0.259
38	UFD1L	1.816	0.226
39	USP1	1.814	0.214
40	VCPIP1	1.813	0.208
41	PSMD14	1.811	0.197
42	UBL4A	1.801	0.131
43	UCHL5	1.800	0.123

LPS + Nigericin

Rank	Gene	Normalized IL1b	z-score
1	STAMBP	3.064	2.238
2	USP39	2.966	2.015
3	ZRANB1	2.924	1.917
4	USP17L2	2.899	1.861
5	USP38	2.875	1.806
6	USP47	2.846	1.740
7	USP25	2.846	1.739
8	UBTD1	2.794	1.620
9	UCK2	2.775	1.576
10	UCHL3	2.727	1.465
11	COPS5	2.698	1.399
12	USP14	2.689	1.379
13	USP8	2.687	1.375
14	USP33	2.665	1.323
15	ATXN3	2.656	1.304
16	USP31	2.608	1.194
17	UBTD2	2.606	1.187
18	USP37	2.518	0.986
19	USP43	2.515	0.981
20	OTUD1	2.514	0.976
21	USP34	2.505	0.957
22	USP5	2.476	0.890
23	FBXO8	2.452	0.834
24	USP3	2.449	0.828
25	USP22	2.433	0.792
26	STAMBPL1	2.397	0.709
27	BRCC3	2.389	0.691
28	USP42	2.366	0.637
29	USP32	2.357	0.617
30	USP24	2.340	0.579
31	USPL1	2.320	0.532
32	YOD1	2.315	0.521
33	USP54	2.313	0.516
34	JOSD2	2.293	0.471
35	UCHL5	2.272	0.422
36	USP30	2.254	0.381
37	USP40	2.237	0.342
38	USP27X	2.233	0.332
39	USP26	2.206	0.271
40	PRPF8	2.200	0.258
41	UFD1L	2.189	0.233
42	USP35	2.184	0.220
43	OTUD4	2.153	0.150

LPS

Rank	Gene	Normalized IL1b	z-score
44	UBTD2	1.796	0.102
45	USP39	1.791	0.067
46	USP29	1.789	0.059
47	BRCC3	1.785	0.032
48	USP28	1.767	-0.077
49	USP32	1.763	-0.107
50	USP19	1.754	-0.164
51	USP7	1.752	-0.173
52	USP41	1.748	-0.200
53	MPND	1.745	-0.214
54	USP34	1.744	-0.220
55	UBTD1	1.738	-0.260
56	USP4	1.738	-0.262
57	USP10	1.736	-0.273
58	USP25	1.730	-0.307
59	USP44	1.729	-0.317
60	USP9Y	1.729	-0.317
61	FBXO8	1.722	-0.362
62	USP12	1.721	-0.368
63	YOD1	1.721	-0.369
64	USP15	1.718	-0.388
65	UBL5	1.716	-0.397
66	USP22	1.715	-0.405
67	PARP11	1.714	-0.408
68	USP30	1.713	-0.416
69	USP49	1.710	-0.436
70	UEVLD	1.710	-0.437
71	OTUB1	1.699	-0.501
72	USP17L2	1.692	-0.545
73	ATXN3	1.690	-0.557
74	UBL3	1.689	-0.563
75	USP47	1.683	-0.603
76	USP14	1.680	-0.619
77	USP42	1.680	-0.622
78	USP54	1.678	-0.634
79	FBXO7	1.672	-0.671
80	USP35	1.660	-0.743
81	USP16	1.657	-0.765
82	USP53	1.654	-0.781
83	USP2	1.654	-0.782
84	USP31	1.626	-0.956
85	BAP1	1.626	-0.959
86	USP51	1.617	-1.017
87	USP37	1.616	-1.022
88	OTUD1	1.578	-1.258

LPS + Nigericin

Rank	Gene	Normalized IL1b	z-score
44	USP15	2.146	0.133
45	USP36	2.126	0.087
46	PARP11	2.098	0.023
47	USP18	2.082	-0.014
48	USP2	2.063	-0.058
49	PAN2	1.986	-0.234
50	UBL5	1.869	-0.501
51	USP1	1.862	-0.518
52	BAP1	1.826	-0.601
53	USP48	1.822	-0.609
54	SENP2	1.814	-0.627
55	USP9Y	1.796	-0.670
56	OTUD6B	1.796	-0.670
57	FBXO7	1.792	-0.679
58	USP16	1.785	-0.694
59	USP21	1.777	-0.712
60	USP29	1.769	-0.733
61	USP50	1.753	-0.768
62	UBL4A	1.747	-0.781
63	USP46	1.741	-0.795
64	CYLD	1.739	-0.800
65	USP28	1.739	-0.801
66	USP45	1.736	-0.808
67	OTUB2	1.730	-0.820
68	MYSM1	1.726	-0.829
69	USP49	1.715	-0.855
70	USP12	1.714	-0.858
71	UBL3	1.683	-0.930
72	USP44	1.679	-0.938
73	OTUD7A	1.675	-0.948
74	TNFAIP3	1.668	-0.963
75	PSMD14	1.667	-0.965
76	USP20	1.664	-0.972
77	USP41	1.664	-0.972
78	USP4	1.664	-0.973
79	USP19	1.660	-0.981
80	MPND	1.660	-0.981
81	OTUD5	1.658	-0.987
82	USP9X	1.657	-0.989
83	USP53	1.654	-0.996
84	USP13	1.648	-1.010
85	JOSD1	1.647	-1.011
86	VCPIP1	1.640	-1.028
87	USP7	1.622	-1.068
88	UCHL1	1.617	-1.079

LPS

Rank	Gene	Normalized IL1b	z-score
89	OTUD4	1.577	-1.262
90	UBR1	1.520	-1.617
91	UCK2	1.513	-1.663
92	USP9X	1.483	-1.845
93	USP20	1.453	-2.034
94	USP36	1.447	-2.074
95	UCHL1	1.413	-2.286
96	USP26	1.316	-2.887

LPS + Nigericin

Rank	Gene	Normalized IL1b	z-score
89	UEVLD	1.617	-1.081
90	OTUB1	1.613	-1.088
91	USP11	1.605	-1.108
92	USP51	1.600	-1.118
93	OTUD7B	1.587	-1.149
94	UBR1	1.548	-1.238
95	USP6	1.547	-1.240
96	USP10	1.539	-1.259

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