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Bénédicte F. Py, Mi-Sung Kim, Helin Vakifahmetoglu-Norberg, Junying Yuan. Deubiquitination of NLRP3 by BRCC3 Critically Regulates Inflammasome Activity. *Molecular Cell*, 2013, 49 (2), pp.331-338. 10.1016/j.molcel.2012.11.009 . inserm-03938600

**HAL Id: inserm-03938600**

**<https://inserm.hal.science/inserm-03938600>**

Submitted on 13 Jan 2023

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# **Deubiquitination of NLRP3 by BRCC3 critically regulates the inflammasome activity**

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Running title

BRCC3 regulates NLRP3 inflammasome activity

## Summary

NLRP3 is an important pattern recognition receptor involved in mediating inflammasome activation in response to viral and bacterial infections as well as various pro-inflammatory stimuli associated with tissue damage or malfunction. Upon activation, NLRP3 assembles a multimeric inflammasome complex comprising the adaptor ASC and the effector pro-caspase-1 to mediate the activation of caspase-1. Although NLRP3 expression is induced by the NF- $\kappa$ B pathway, the post-transcriptional molecular mechanism controlling the activation of NLRP3 remains elusive. Using both pharmacological and molecular approaches, we show that the activation of NLRP3 inflammasome is regulated by a deubiquitination mechanism. We further identify the deubiquitinating enzyme, BRCC3, as a critical regulator of NLRP3 activity by promoting its deubiquitination and characterized NLRP3 as the first substrate for the cytosolic BRCC3-containing BRISC complex. Our results elucidate a novel regulatory mechanism, involving BRCC3-dependent NLRP3 regulation and highlight NLRP3 ubiquitination as a new potential therapeutic target for inflammatory diseases.

## Highlights

- A small molecule DUB inhibitor G5 inhibits inflammasome activation
- G5 induces NLRP3 poly-ubiquitination
- Deubiquitination of NLRP3 regulates the activation of inflammasome
- The deubiquitinating enzyme BRCC3 deubiquitinates the LRR domain of NLRP3

Key words

NLRP3, inflammasome, BRCC3, ubiquitin, caspase-1.

## Introduction

The innate immunity system constitutes a highly efficient barrier to diverse organismal insults by rapidly detecting pathogens as well as tissue damage. The activation of pattern recognition receptors (PRRs) by pathogen- and damage-associated molecular patterns (PAMPs and DAMPs) leads to the early local detection of the insult and production of pro-inflammatory cytokines. NLRP3 is an important PRR involved in anti-viral and anti-bacterial innate immunity as well as adjuvant properties linking innate to adaptive immunity. On the other hand, inappropriate activation of NLRP3 contributes to deleterious inflammatory syndromes including hereditary periodic fever syndromes (HPF) associated with substitution mutations in the NLRP3 gene, LPS-induced septic shock, gout disease, atherosclerosis, diabetes, Alzheimer's disease and ischemia-reperfusion injuries (Martinon et al., 2009). Upon activation, NLRP3 assembles a typical multimeric inflammasome complex comprising the adaptor ASC and the effector pro-caspase-1 to mediate the activation of caspase-1. NLRP3 is activated downstream of numerous stimuli such as bacterial toxins like nigericin, extracellular ATP, crystals such as silica, amyloid- $\beta$ , hyperglycemia and alum.

Caspase-1 is an important pro-inflammatory caspase directly involved in processing and release of cytosolic pro-cytokines, including pro-IL-1 $\beta$  and IL-18 (Keller et al., 2008; Martinon et al., 2009). Activation of caspase-1 is a critical downstream functional output of the inflammasome. In some conditions, caspase-1 triggers pyroptosis, a highly inflammatory form of cell death, which shares features with both apoptosis and necrosis.

Consistent with the deleterious consequences of its inappropriate activation, the inflammasome is tightly regulated. NLRP3 gene expression is inducible downstream of NF- $\kappa$ B activators and requires prior sensitization by TLR ligands in macrophages and dendritic cells (Bauernfeind et al., 2009). However, post-transcriptional regulations of NLRP3 are much less well characterized. Several decoy interactors such as PYD- and CARD-only proteins may interfere with the inflammasome assembly (Stehlik and Dorfleutner, 2007). Dynamic post-translational modification of proteins with covalent ubiquitin chains regulates an assortment of signaling pathways through proteasome- or autophagy-mediated degradation or by altering protein activity and/or localization. Ubiquitin conjugation is mediated via an E1-E2-E3 cascade whereas ubiquitin removal is catalyzed by deubiquitinating enzymes (DUBs). Murine BRCC3 and its human homolog BRCC36 (referred to as BRCC3 thereafter) are JAMM domain-containing  $\text{Zn}^{2+}$  metalloprotease DUBs. BRCC3 is known to participate in DNA damage response in the BRCA1-A complex recruited by the chromatin at double strand break sites, as well as in the cytosolic BRISC complex (Cooper et al., 2009; Shao et al., 2009). Neither biological function nor direct substrates have been identified so far for the cytosolic DUB activity of BRCC3.

Since modulating the inflammasome activity presents high therapeutic potential (Lopez-Castejon and Pelegrin, 2012), we explored the mechanism of NLRP3 regulation using a combination of pharmacological and molecular approaches. We discovered that G5, a small molecule inhibitor of DUBs, inhibits the deubiquitination and subsequent activation of NLRP3, suggesting that NLRP3 activity is regulated by its ubiquitination. We next screened an expression library for DUBs and identified BRCC3 as an

endogenous DUB regulating NLRP3. Our results reveal a novel pathway that regulates the NLRP3 inflammasome and identify NLRP3 as the first cytosolic substrate for BRCC3.

## Results

### **A small molecule DUB inhibitor G5 inhibits IL-1 $\beta$ secretion**

In order to identify regulatory mechanisms that control inflammasome activity, we screened for compounds that can inhibit IL-1 $\beta$  secretion in LPS-primed murine inflammatory peritoneal macrophages treated with ATP. An isopeptidase inhibitor G5 (3,5-bis((4-Nitrophenyl)methylene)-1,1-dioxide, tetrahydro-4H-thiopyran-4-one, Figure S1A) (Aleo et al., 2006) inhibited IL-1 $\beta$  secretion when added at the same time as LPS as well as when added during the last 30 min simultaneously with ATP (Figure 1A). Similar results were obtained when IL-1 $\beta$  secretion was triggered by the bacterial toxin nigericin (Figure 1B). The IC<sub>50</sub> value of G5 to inhibit ATP-triggered IL-1 $\beta$  secretion was estimated to be ~0.2  $\mu$ M (Figure 1C).

### **Selective inhibition of the NLRP3 inflammasome by G5**

Next, we tested whether G5 could inhibit the LPS-triggered TLR4-dependent transcription response or the NLRP3-mediated inflammasome activation. G5 had no effect on LPS-induced I $\kappa$ B phosphorylation and degradation (Figure S1B), nor did it decrease the inflammasome-independent TNF- $\alpha$  secretion (Figure S1C). These data show that G5 did not inhibit the TLR4-dependent transcription response. To assess the effect of G5 on the NLRP3 inflammasome activation, we used NG5 cells, an immortalized NLRP3 KO macrophage line stably expressing Flag-NLRP3 (Juliana et al., 2010). Treatment of NG5 cells with ATP is sufficient to trigger NLRP3 inflammasome complex assembly and caspase-1 cleavage and secretion independently of any TLR-dependent signaling. Treatment with G5 significantly inhibited the cleavage and secretion of caspase-1



triggered by ATP in NG5 cells (Figure 2A), suggesting that the inhibitory effect of G5 is on the activation of inflammasome. Consistently, in LPS-primed primary macrophages, G5 had no effect on the induction of pro-IL-1 $\beta$ , but inhibited caspase-1 p20 cleavage and secretion, as well as mature IL-1 $\beta$  p17 secretion triggered by extracellular ATP (Figure 2B). Since ASC oligomerization induced by ATP is an important step in the activation of caspase-1, we determined the effect of G5 on the formation of multimeric ASC in LPS-primed macrophages. Indeed, we found that G5 strongly inhibited the formation of ASC multimers in LPS/ATP-treated macrophages (Figure 2C). Thus, we conclude that G5 can inhibit the inflammasome assembly triggered by PRR.

To explore further the effect of G5 on inflammasome response, we examined the effect of G5 on additional NLRP3 inflammasome activators, including nigericin, the lysomotropic agent HLLoMe and silica crystals (Hornung et al., 2008). We found that G5 inhibited the cleavage of caspase-1 and the release of mature IL-1 $\beta$  after the treatment of all of these agents (Figure 2D-H). Notably, however, G5 inhibitory activity is selective for NLRP3-dependent inflammasome stimuli, as G5 had no effect on caspase-1 cleavage and mature IL-1 $\beta$  secretion triggered by *S. typhimurium* or cytosolic polydAdT DNA which are dependent on the NLRC4 and AIM2 inflammasomes, respectively, and independent of NLRP3 (Figure S2A-F) (Fernandes-Alnemri et al., 2009; Mariathasan et al., 2004). Consistently, the treatment of G5 does not reduce expression level of common components of the NLRP3, AIM2 and NLRC4 inflammasome namely ASC and pro-caspase-1 (Figure S2G)

### **G5 triggers NLRP3 ubiquitination**

The ability of G5, a known deubiquitinating enzyme inhibitor, to selectively inhibit the NLRP3 inflammasome activity implicates ubiquitination in regulating NLRP3 activation. Consistent with this possibility, in NG5 cells, G5 treatment triggered the appearance of high apparent molecular weight NLRP3 species that reacted with anti-ubiquitin (Figure 3A-B). However, the ubiquitination levels of NLRP3 in G5-treated cells did not increase further upon the addition of MG132 or E64d, inhibitors of proteasome- and autophagy-mediated proteolysis, respectively, suggesting that G5-triggered NLRP3 association with ubiquitin did not promote its degradation by either mechanism. Similarly, we found that G5 also induced the appearance of high molecular weight endogenous NLRP3 species that could react with anti-ubiquitin in LPS-primed macrophages, but not in unstimulated macrophages that do not express NLRP3, demonstrating the specificity of G5-mediated ubiquitination (Figure 3C). Similarly to our observation in NG5 cells, the treatment of G5 also led to the appearance of high molecular weight endogenous NLRP3 species (Figure 3D). Since co-treatments with MG132 or E64d together with G5 did not increase these G5-induced high molecular weight NLRP3 species, we conclude that the G5-induced ubiquitination is unlikely to function in directing endogenous NLRP3 to proteasome or autophagy-mediated degradation.

To test whether G5-induced high molecular weight NLRP3 species is a result of its polyubiquitination, we immunoprecipitated NLRP3 in denaturing conditions from lysates of 293T cells expressing His-Ubiquitin and Flag-NLRP3 that were treated with G5 or vehicle control. As shown in Figure 3E, the treatment of G5 rapidly increased the levels of ubiquitinated NLRP3. Taken together, we conclude that higher molecular

weight ubiquitin-positive species after immunoprecipitation with anti-NLRP3 in G5 treated macrophages most likely represent polyubiquitinated NLRP3.

We then analyzed the type of ubiquitin chains and the ubiquitinated domain of NLRP3 following G5 treatment. Both anti-K48 and anti-K63 ubiquitin antibodies can react with immunoprecipitated NLRP3 in NG5 macrophages after G5 treatment, suggesting that G5-induced ubiquitination of NLRP3 carries mixed K48 and K63 ubiquitin chains (Figure 3F). Consistent with this possibility, neither HA-UbiK48only nor HA-UbiK63only expressions alone blocked G5-induced Flag-NLRP3 ubiquitination in 293T cells (Figure 3G). We also examined the effects of G5 on ubiquitination of different domains of NLRP3. The treatment of G5 increased the ubiquitination of overexpressed  $\Delta$ PYD,  $\Delta$ LRR mutants of NLRP3 as well as NACHT and LRR domains alone, but not the PYD domain of NLRP3 in 293T cells (Figure 3H). Taken together, these data suggest that inhibition of deubiquitination by G5 triggers the polyubiquitination of both the NACHT and LRR domains of NLRP3 with mixed K63 and K48 chains which critically inhibits NLRP3 inflammasome activation.

### **Identification of DUBs regulating the NLRP3 inflammasome**

We then searched for the deubiquitinating enzyme involved in the regulation of NLRP3 ubiquitination by screening a library of DUB expression vectors (Sowa et al., 2009). We first determined the ability of Flag-NLRP3 to co-immunoprecipitate with 74 Myc-tagged human DUBs expressed in 293T cells, in which 43 DUBs scored positive (Figure S3A, Table S1). We then determined if overexpression of these 43 DUBs might reduce the levels of NLRP3 ubiquitination and found that 12 DUBs, out of the 43 DUB genes

screened, scored positive. Finally, we screened if knockdown of any of these 12 DUBs might block IL-1 $\beta$  secretion triggered by ATP in LPS-primed macrophages. We found that the downregulation of BRCC3 led to a specific and strong blockage of IL-1 $\beta$  release triggered by ATP, while had no effect on the NLRP3-independent TNF- $\alpha$  and IL-6 secretion induced by LPS (Figure S3B). Therefore, we conclude that BRCC3 might be involved in regulating deubiquitination of NLRP3.

### **BRCC3 promotes the inflammasome activation by deubiquitinating NLRP3**

Consistent with an activator of NLRP3 activity, knockdown of BRCC3 inhibited mature IL-1 $\beta$  and activated caspase-1 secretion triggered by ATP without affecting pro-IL-1 $\beta$  induction in LPS-primed macrophages (Figure 4A, S4). In addition, BRCC3 knockdown inhibited NLRP3-dependent TLR-independent cleaved caspase-1 secretion triggered by ATP in NG5 cells (Figure 4B, S4). These results demonstrate that BRCC3 is required for inflammasome activation but dispensable for LPS-induced transcriptional response. Since transduction of WT BRCC3, but not of the inactive mutant BRCC3 H122Q, rescued caspase-1 cleavage and IL-1 $\beta$  secretion inhibited by BRCC3 KD (Figure 4C), we conclude that BRCC3 regulates NLRP3 through its DUB activity. Consistent with a regulatory role of BRCC3 with NLRP3 inflammasome, we found that endogenous BRCC3 co-immunoprecipitated with Flag-NLRP3 from NG5 cell lysates (Figure 4D), and with endogenous NLRP3 from LPS-primed and G5-treated macrophages (Figure 4E), suggesting that BRCC3 binds to ubiquitinated NLRP3. Moreover, BRCC3 knockdown induced ubiquitination of Flag-NLRP3 in NG5 cells (Figure 4F), and the ubiquitination of endogenous NLRP3 in LPS-primed inflammatory peritoneal

macrophages (Figure 4G). In addition, consistent with its role as a deubiquitination enzyme for NLRP3, BRCC3 expression significantly reduced the extent of NLRP3 covalent ubiquitination in 293T (Figure 4H).

We then mapped the NLRP3 domain targeted by BRCC3. Both the NACHT oligomerization domain and the LRR sensor domain, but not the adaptor recruitment PYD domain, independently co-immunoprecipitated with BRCC3 (Figure 4I-J). Notably, G5 dramatically increased BRCC3 binding to high molecular weight ubiquitinated LRR, suggesting that BRCC3 specifically interacts with ubiquitinated LRR domain. Consistently, the expression of Myc-BRCC3 in 293T cells effectively blocked spontaneous ubiquitination of overexpressed LRR as observed by ubiquitin co-immunoprecipitation with NLRP3 assay (Figure 4K). Consistently, LRR ubiquitination were restored in the presence of G5, further supporting the inhibitory effect of G5 on BRCC3-mediated deubiquitination of NLRP3 (Figure 4K). Altogether these results demonstrate that BRCC3 mediates the deubiquitination of the LRR domain of NLRP3, an important regulatory mechanism in the activation of NLRP3 inflammasome by its cognate stimuli.

## Discussion

In this study we describe a novel pathway regulating the NLRP3 inflammasome by deubiquitination. The use of G5, a small molecule inhibitor of deubiquitination, in a pharmacological approach, provides the first hint for the involvement of deubiquitination in regulating the activity of the NLRP3 inflammasome and the motivation to search for the deubiquitinating enzyme involved. By screening a DUB library, we identify BRCC3 as a novel regulator of NLRP3 ubiquitination and activity. The identification of a novel deubiquitination pathway regulating NLRP3 constitutes an important advance in the field of inflammation with therapeutic potential since NLRP3 plays pivotal roles in many inflammatory disorders. Since the current therapies are solely based on neutralizing proinflammatory cytokines in the circulation, targeting of ubiquitination of NLRP3 may provide a new approach for the treatment of inflammatory diseases (Lopez-Castejon and Pelegrin, 2012).

The precise mechanism of NLRP3 activation by PAMPs and DAMPs is unclear as no direct binding of these agonists to NLRP3 has been reported. The structural diversity of PAMPs and DAMPs suggests that they may trigger intracellular changes that in turn converge on a common activation mechanism of NLRP3 (Martinon et al., 2009). Since G5 inhibits inflammasome activation triggered by diverse kinds of NLRP3 agonists including  $K^+$  homeostasis-, ROS- or cathepsin-dependent stimuli, but not agonists of the NLRC4 and AIM2 inflammasomes which share common downstream constituents with the NLRP3 inflammasome, the most likely hypothesis is that G5 blocks the activation of NLRP3 itself. Our study suggests the ubiquitination of NLRP3 as critically involved in regulating the inflammasome activation.

We identify BRCC3 as a critical regulator of NLRP3 ubiquitination and inflammasome activity. BRCC3 together with BRE and HSPC142 compose the nuclear BRCA1-A complex in association with ABRAXAS (Shao et al., 2009), and the cytosolic BRISC complex in association with ABRO1 (Cooper et al., 2009). Since the BRISC complex is involved in tissue damage after artery occlusion-reperfusion (Cilenti et al., 2011), we hypothesize that the BRISC complex may facilitate myocardial infarction injury through the deubiquitination and activation of NLRP3 which has been proposed to mediate necrotic cell sensing and the subsequent release of the proinflammatory cytokine IL-1 $\beta$  after hypoxic injury (Iyer et al., 2009). Noteworthy, BRCC3 is also regulated at the transcriptional level downstream of the MAPK pathway (Boudreau et al., 2007). Many TLR ligands including LPS may potentially upregulate BRCC3 expression through the MAPK pathway and macrophages priming may not only be necessary for NLRP3 expression (Bauernfeind et al., 2009) but also favor its deubiquitination and subsequent activation.

Since BRCC3 deubiquitinates the NLRP3 LRR domain and none of the substitution mutations in the NLRP3 gene associated with HPF and identified to date alter the LRR domain, our results do not support the notion that defects in NLRP3 ubiquitination may be related to the constitutive NLRP3 activation in these patients (Shinkai et al., 2008). Inhibiting NLRP3 inflammasome activation may have beneficial effect in a large set of diseases including ischemia, Alzheimer's disease, diabetes and metabolic syndrome, gout disease, atherosclerosis, cancer, trauma as well as bacterial and viral infection infections, as suggested by the clinical benefits obtained with IL-1R antagonists in several of these pathologies (Lopez-Castejon and Pelegrin, 2012).

Conversely, the adjuvant properties of NLRP3 inflammasome activation can be exploited (Ghiringhelli et al., 2009). Our discovery of a novel BRCC3-dependent mechanism that controls NLRP3 inflammasome may be instrumental in developing specific inhibitors and activators serving as new therapeutic modalities against the growing number of pathologies associated with inappropriate activation of the NLRP3 inflammasome.



## Materials and Methods

### Mice

C57B6 mice were housed at the W. Alpert Animal Facility (Harvard Medical School). Experiments were performed in accordance with federal and institutional guidelines. Inflammatory peritoneal macrophages were elicited by the intraperitoneal injection of 4% thioglycolate broth for 4 days.

### Cell growth conditions

Inflammatory peritoneal macrophages were cultured at  $0.5-1 \times 10^6$  cells/ml in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 1X penicillin/streptomycin (PS) and 10 % Fetal Bovine Serum (FBS) (Gibco). For trichloroacetic acid (TCA)-precipitation assay and *S. typhimurium* infection, cells were cultured without FBS and PS respectively.

### Inflammasome activation

*S. typhimurium* strain 1344 was provided by M. Starnbach (Harvard Medical School, Boston). Bacteria grown in LB in a log phase were resuspended in PBS and added to the macrophages. PolydAdT was transfected using Lipofectamine 2000 (Invitrogen).

### Constructs

Flag-NLRP3 and its deletion mutants ( $\Delta$ PYD,  $\Delta$ LRR, NACHT, LRR, PYD) were a gift from J. Tschopp and F. Martinon (Lausanne University, Switzerland). The expression vectors of His-Ubiquitin, HA-Ubiquitin, HA-Ubiquitin K48-only and HA-Ubiquitin K63-

only were obtained from A. Goldberg (Harvard Medical School, Boston). The Myc-DUB library was a gift from W. Harper (Harvard Medical School, Boston). The pOZ-BRCC3 and pOZ-BRCC3 H122Q constructs were shared by E. Cooper (Hartwick College, Oneonta).

#### siRNA

siRNAs were ordered as SMARTpool (Dharmacon). Inflammatory macrophages were transfected in 96-well format using  $16 \times 10^{-12}$  mol siRNA and 0.15  $\mu$ l Lipofectamine RNAiMax (Invitrogen) in DMEM + 10 % FBS. Media was changed 6 h later and cells were cultured for 48 h in DMEM + 10 % FBS + ATB.

#### Reagents

The following reagent were used: G5 (EMD), LPS from Escherichia coli 0111:B4 (Sigma), ATP (Sigma), nigericin (Sigma), silica (U.S.Silica), HLLOMe (Chem-Impex Intl), polydAdT (InVivogen), MG132 (Sigma), E64d (Enzo Life Science), complete protease inhibitor cocktail tablets (Roche), disuccinimidyl suberate (DSS, Thermo Scientific), protein A/G UltraLink resin (Thermo scientific), anti-Flag M2 and anti-c-Myc agarose beads (Sigma).

#### ELISA test

Anti-mIL-1 $\beta$ , anti-mTNF- $\alpha$  and anti-IL-6 ELISA assays were performed using the DuoSet ELISA kits (R&D Systems).

## Western-blot analysis

Proteins from cell media were TCA-precipitated. Cells were lysed in Laemmli buffer 2 X. Samples were analyzed by SDS-PAGE and transferred to PVDF membranes. The following antibodies were used: anti-actin (Millipore), anti-NLRP3 (adipogen), anti-I $\kappa$ B (Santa Cruz), anti-phospho(Ser52)-I $\kappa$ B (Cell signaling), anti-laminB (Santa Cruz), anti-caspase-1 (in house), anti-IL-1 $\beta$  (R&D systems), anti-ubiquitin (Dako), anti-Flag M2 (Sigma), anti-c-Myc (Invitrogen), Apu2.07 anti-K48- and Apu3.A8 anti-K63-linked polyubiquitin antibodies were a gift from Vishva Dixit (Genentech) (Newton et al., 2008) and secondary HRP-conjugated antibodies (SouthernBiotech).

## Immunoprecipitation

To detect NLRP3 ubiquitination, cells were lysed in RIPA buffer (Tris HCl pH 8.0 50 mM, NaCl 150 mM, EDTA 1 mM, sodium deoxycholate 0.05 %, Triton X-100 1 %, SDS 0.1 %, PMSF 1 mM, protease inhibitors). Flag-NLRP3 was immunoprecipitated with anti-Flag M2 beads. Endogenous NLRP3 was immunoprecipitated with anti-NLRP3 (adipogen) and protein A/G resin. For co-immunoprecipitation assay, cells were lysed in NP-40 buffer (Tris HCl pH7.6 50 mM, NaCl 150 mM, EDTA 5 mM, NP-40 1%, PMSF 1 mM, protease inhibitors). Myc-DUBs were immunoprecipitated with anti-c-Myc beads. Immunoprecipitated complexes were washed in lysis buffer and proteins eluted in Laemmli buffer 2 X. To test covalent binding between NLRP3 and ubiquitin, cells were lysed in denaturing buffer (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM Tris-HCl pH 8.0, 500 mM NaCl, 10 % glycerol, 0.1 % triton X-100, 10 mM  $\beta$ -mercaptoethanol, 10 mM imidazole) and His-ubiquitin was pull-downed with Ni-NTA beads (Qiagen). Beads were

rinsed with lysis buffer (30 mM imidazole), and eluted (0.15 M Tris-HCl pH 6.7, 5 % SDS, 30 % glycerol, 200 mM imidazole, 0.72 M  $\beta$ -mercaptoethanol).

#### ASC oligomerisation assay

Macrophages were lysed in buffer A (Hepes 20 mM, KCl 10 mM,  $\text{MgCl}_2$  1.5 mM, EDTA 1 mM, protease inhibitors) by shearing 20 times through a 28-gauge needle. Intact cells and nuclei were pelleted by centrifugation (340 g, 8 min), and CHAPS buffer (Hepes 20 mM,  $\text{MgCl}_2$  5 mM, EGTA 0.5 mM, CHAPS 0.1%, protease inhibitors) was added to the supernatant. Proteins associated to the pyroptosome were pelleted by centrifugation (2650 g, 8 min), crosslinked with DSS (25 mM, 30 min).

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## Acknowledgment

This was supported in part by the National Institute on Aging (R37 AG012859) to JY. We thank Wade Harper (Harvard Medical School, Boston) for the DUB expression library, Emad Alnemri (Thomas Jefferson University, Philadelphia) for immortalized NLRP3 KO, NG5 and wild-type macrophages, Jürg Tschopp and Fabio Martinon (UNIL, Lausanne) for the Flag-NLRP3 and its deletion mutant constructs, Vishva Dixit (Genentech) for anti-K48- and anti-K63-linked polyubiquitin antibodies, Alfred Goldberg (Harvard Medical School, Boston) for the His-Ubiquitin, HA-Ubiquitin, HA-Ubiquitin K48-only and HA-Ubiquitin K63-only constructs, Eric Cooper (Hartwick College, Oneonta) for the BRCC3 and BRCC3 H122Q constructs, Nobuhiko Kayagaki (Genentech) for anti-ASC antibody, Michael Starnbach (Harvard Medical School, Boston) for *Salmonella typhimurium* strain 1344, Thomas Henry (Lyon University) for advices and discussion, and Dimitry Ofengeim for critical reading of the manuscript.

## Figure legends

### Figure 1. G5 inhibits Il-1 $\beta$ secretion.

(A-B) Macrophages were treated with LPS (100 ng/ml) for 8 h and ATP (2 mM) (A) or nigericin (50  $\mu$ M) (B) for the last 30 min.

(C) Macrophages were treated with LPS (100 ng/ml) for 12 h and ATP (2 mM) for the last 30 min. G5 was added 15 min prior ATP.

### Figure 2. G5 inhibits the NLRP3 inflammasome.

(A) NG5 cells were treated with G5 for 2 h and ATP (2 mM) was added for the last 30 min.

(B-C) LPS-primed macrophages (100 ng/ml, 12 h) were treated with ATP (2 mM, 30 min). G5 (1  $\mu$ M) was added 15 min prior ATP. Cells were lysed and proteins from the pyroptosome-containing pellet were cross-linked with DSS and analyzed by western blotting (C).

(D-E) LPS-primed macrophages (100 ng/ml, 12 h) were treated with nigericin (20  $\mu$ M, 6 h). G5 was added 15 min prior nigericin at the indicated concentrations (D) or 1  $\mu$ M (E).

(F-H) LPS-primed macrophages (100 ng/ml, 12 h) were treated with HLLOMe (1 mM, 30 min) (F, H) or silica (0.5  $\mu$ g/ml, 2 h) (G, H). G5 was added 15 min prior NLRP3 activators at the indicated concentrations (F, G) or 1  $\mu$ M (H).

### Figure 3. G5 triggers the ubiquitination of NLRP3.



(A-B) NG5 macrophages expressing Flag-NLRP3 and control NLRP3 KO immortalized macrophages were treated with G5 (2  $\mu$ M) and MG132 (10  $\mu$ M) (A) or E64d (10  $\mu$ g/ml, 2 h) (B). Flag-NLRP3 immunoprecipitates were analyzed for ubiquitination.

(C) Inflammatory peritoneal macrophages were treated with LPS (100 ng/ml, 8 h) and G5 (2  $\mu$ M, 30 min). Endogenous NLRP3 immunoprecipitates were analyzed for ubiquitination.

(D) Inflammatory peritoneal macrophages were treated with LPS (100 ng/ml, 12 h), G5 (0.5  $\mu$ M), MG132 (20  $\mu$ M) and E64d (10  $\mu$ g/ml) for 2 h. Arrowhead, high molecular weight NLRP3.

(E) 293T cells expressing Flag-NLRP3 and His-Ubiquitin were treated with G5 (0.5  $\mu$ M), MG132 (10  $\mu$ M) and E64d (10  $\mu$ g/ml). Proteins covalently bound to His-Ubiquitin were pulled-down in denaturing condition with Ni-NTA beads (upper panel). Flag-NLRP3 immunoprecipitates in RIPA buffer were analyzed for ubiquitination (lower panel).

(F) NG5 and NLRP3 KO macrophages were treated with G5 (2  $\mu$ M, 2 h). Flag-NLRP3 immunoprecipitates were analyzed for their association with K48 and K63 ubiquitin chains.

(G-H) 293T cells co-expressing Flag-NLRP3 and HA-Ubiquitin, HA-Ubiquitin K48-only or HA-Ubiquitin K63-only (G) or Flag-NLRP3 full-length (FL) and its Flag-tagged isolated domains (H) were treated with G5 (0.5  $\mu$ M, 30 min). Flag-NLRP3 immunoprecipitates were analyzed for ubiquitination.

Figure 4. BRCC3 promotes IL-1 $\beta$  secretion by deubiquitinating the LRR domain of NLRP3.

- (A) Inflammatory peritoneal macrophages transfected with non-targeting (NT) or BRCC3-specific siRNA were treated with LPS (100 ng/ml, 12 h) and ATP (2 mM, 30 min).
- (B) NG5 cells transfected with the indicated siRNA were treated with ATP (2 mM, 30 min).
- (C) Immortalized macrophages transduced with BRCC3 or BRCC3 H122Q expression constructs and transfected with the indicated siRNA were treated with LPS (100 ng/ml, 12 h) and ATP (2 mM, 30 min).
- (D) Co-immunoprecipitation of endogenous BRCC3 with Flag-NLRP3 from NG5 cell lysate. NLRP3 KO macrophages were used as negative control.
- (E) Co-immunoprecipitation of endogenous BRCC3 with endogenous NLRP3 from macrophages treated with LPS (100 ng/ml, 12 h) and G5 (1  $\mu$ M, 1 h).
- (F) NG5 cells were transfected with the indicated siRNA. Flag-NLRP3 immunoprecipitates were analyzed for ubiquitination by WB. G5 treatment and NLRP3 KO macrophages were used as positive and negative controls respectively.
- (G) Inflammatory peritoneal macrophages transfected with the indicated siRNA were treated with LPS (100 ng/ml, 12 h). Endogenous NLRP3 immunoprecipitates were analyzed for ubiquitination.
- (H) 293T cells expressing Flag-NLRP3, His-Ubiquitin and Myc-BRCC3 treated with G5 (0.5  $\mu$ M, 10 min) were lysed in denaturing conditions. Ubiquitinated proteins were pulled-down on Ni-NTA beads.

(I-J) Anti-Myc (I) or anti-Flag (J) immunoprecipitation from lysates of 293T cells expressing Myc-BRCC3 and Flag-NLRP3 (FL) or Flag-tagged NLRP3 isolated domains (NACHT,  $\Delta$ PYD,  $\Delta$ LRR, LRR, PYD), treated with G5 (1  $\mu$ M, 1 h). \*, unspecific bands.

(K) 293T cells expressing Myc-BRCC3 and Flag-NLRP3 LRR or PYD were treated with G5 (1  $\mu$ M, 1 h). Flag-NLRP3 immunoprecipitates were analyzed for ubiquitination. Arrowhead, ubiquitinated LRR.

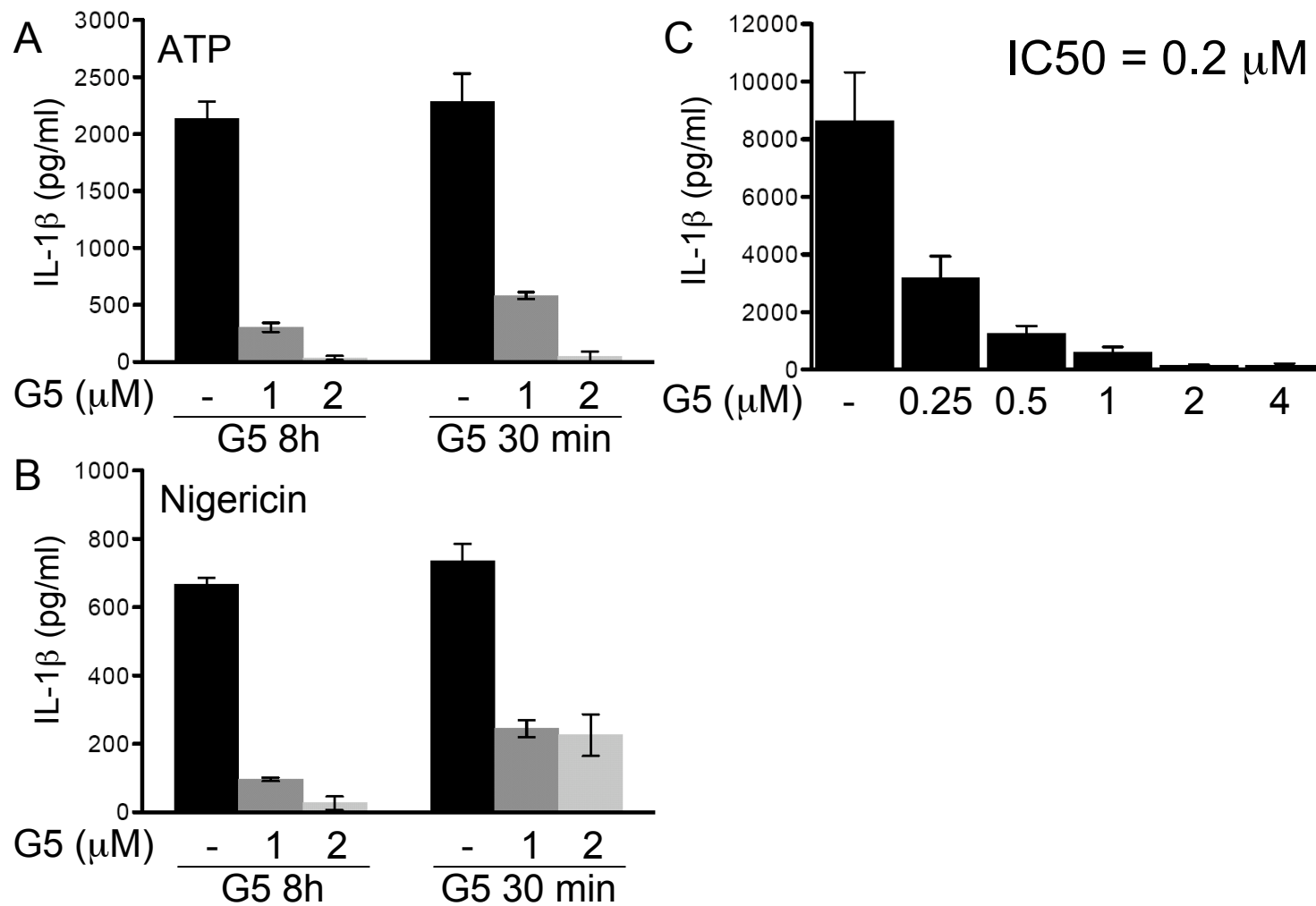


Figure 1

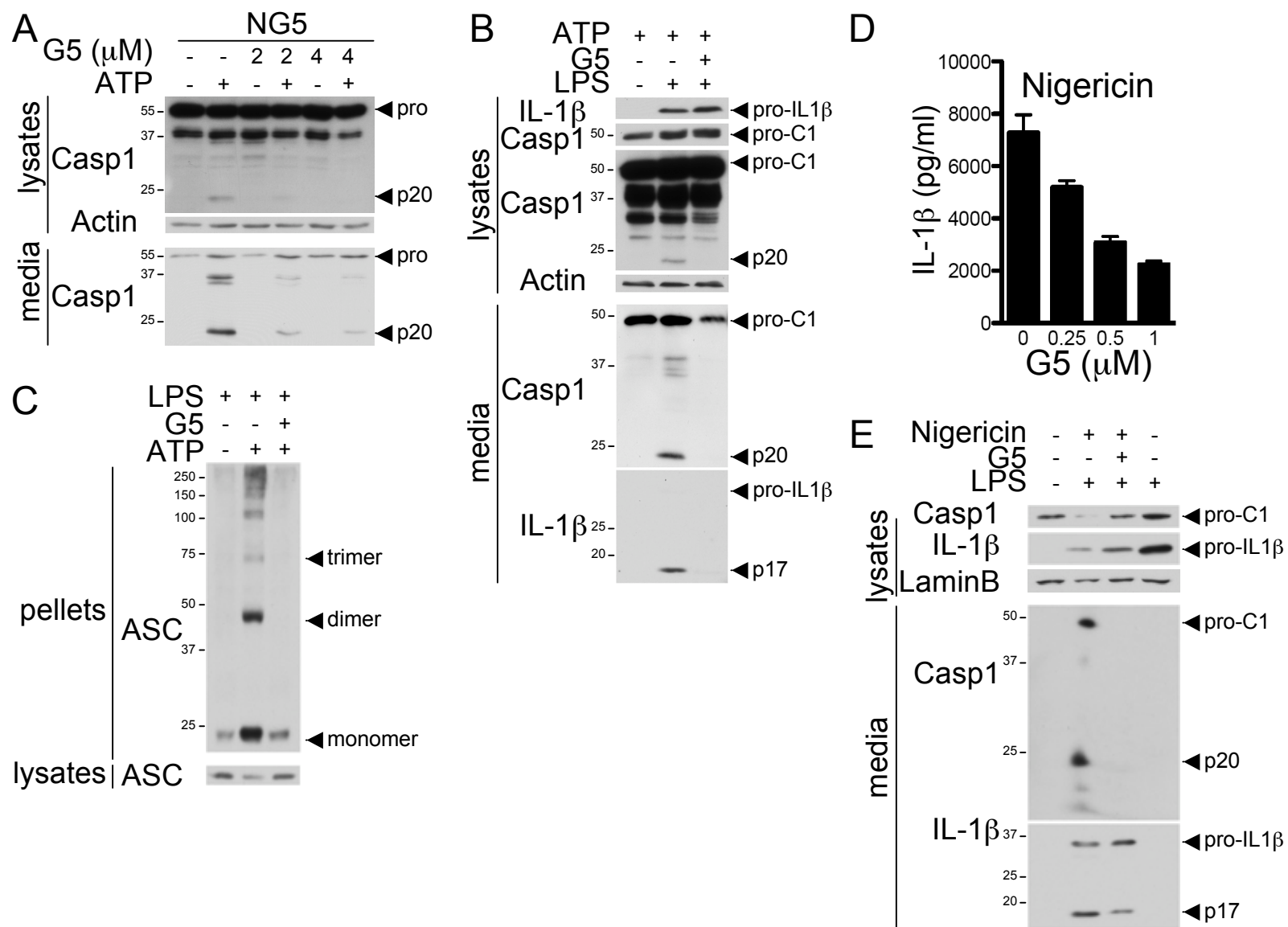


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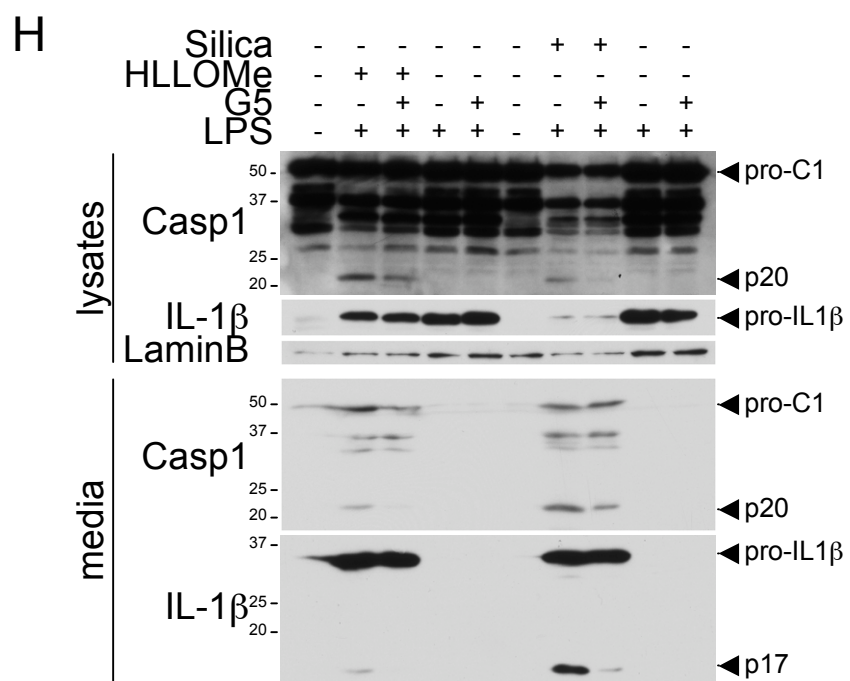
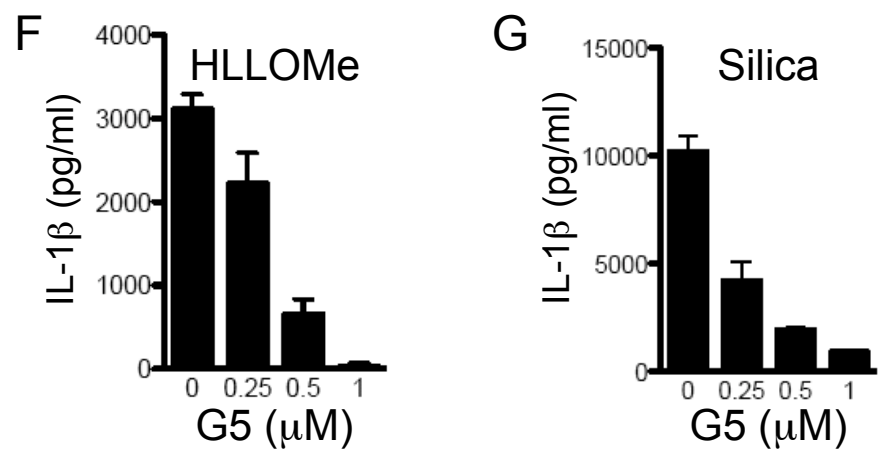


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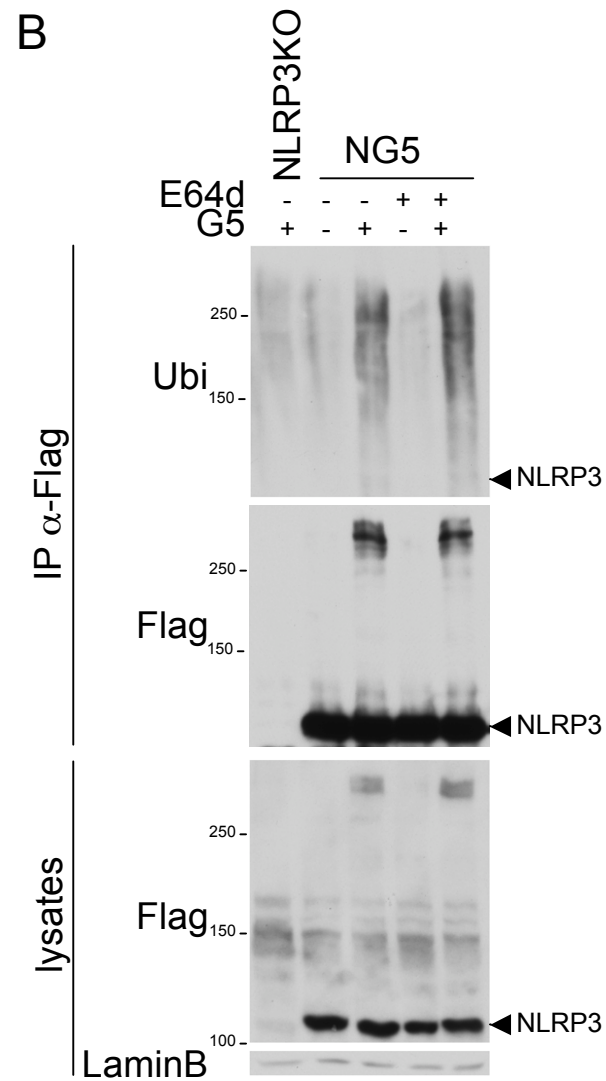
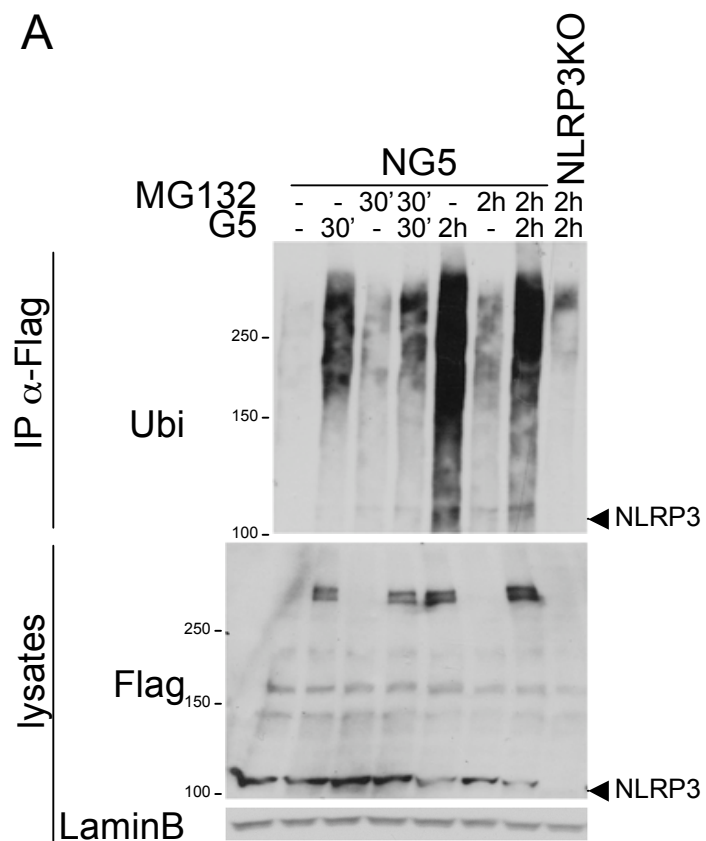


Figure 3

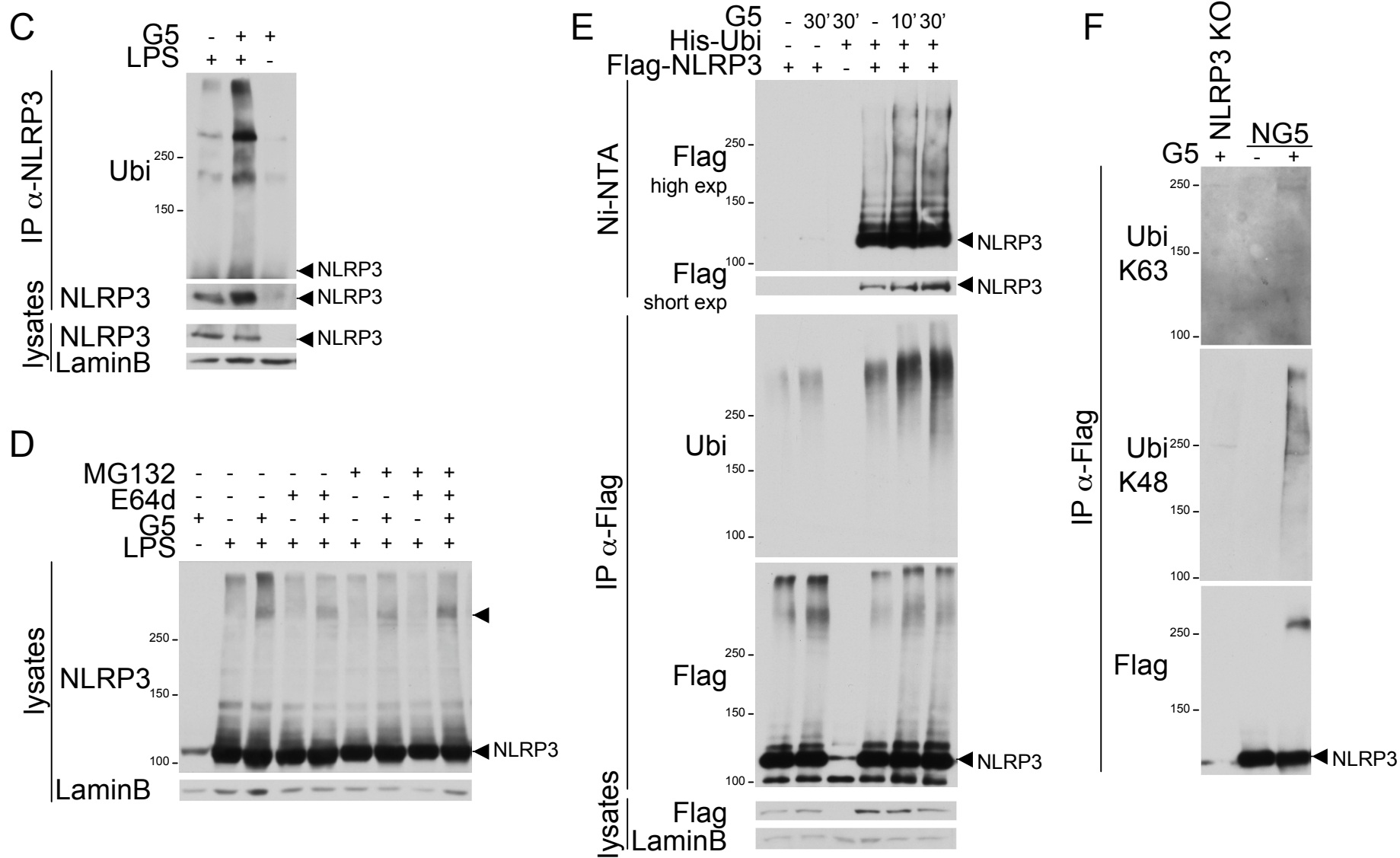


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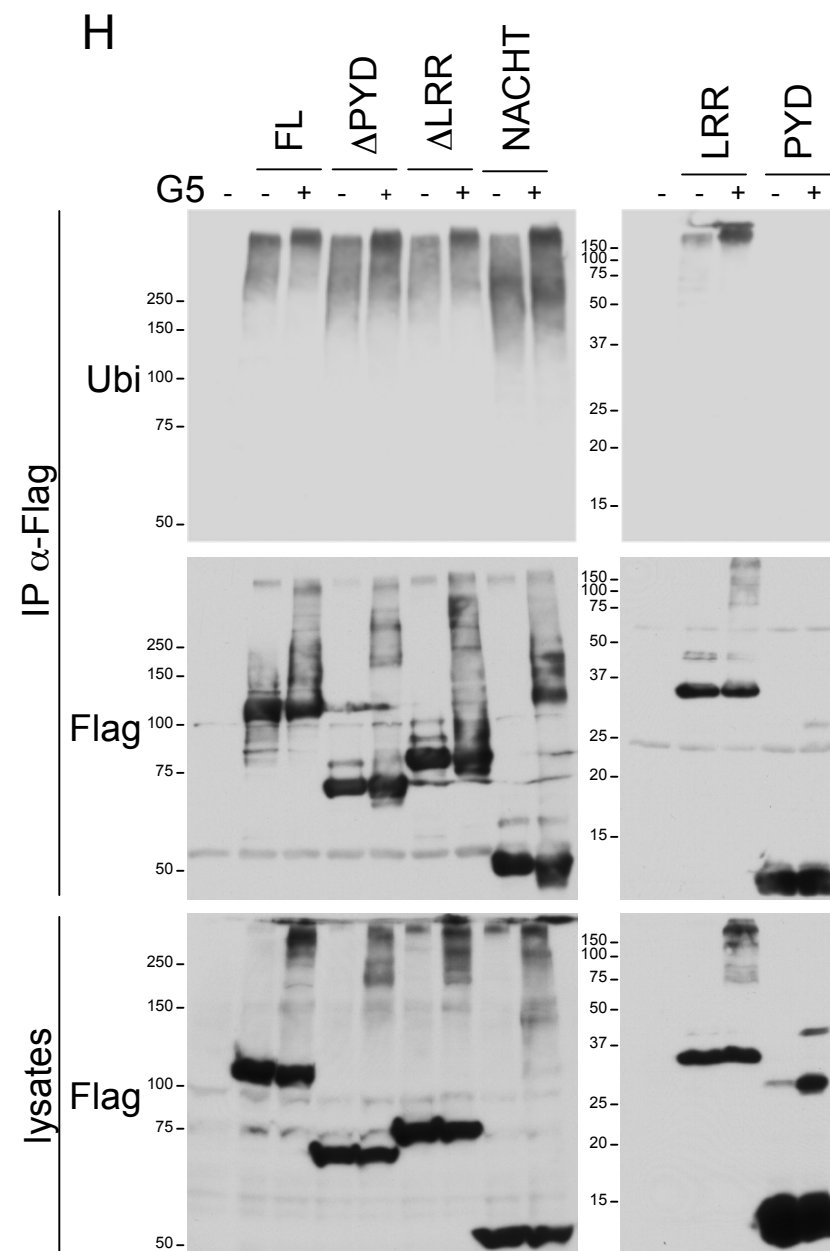
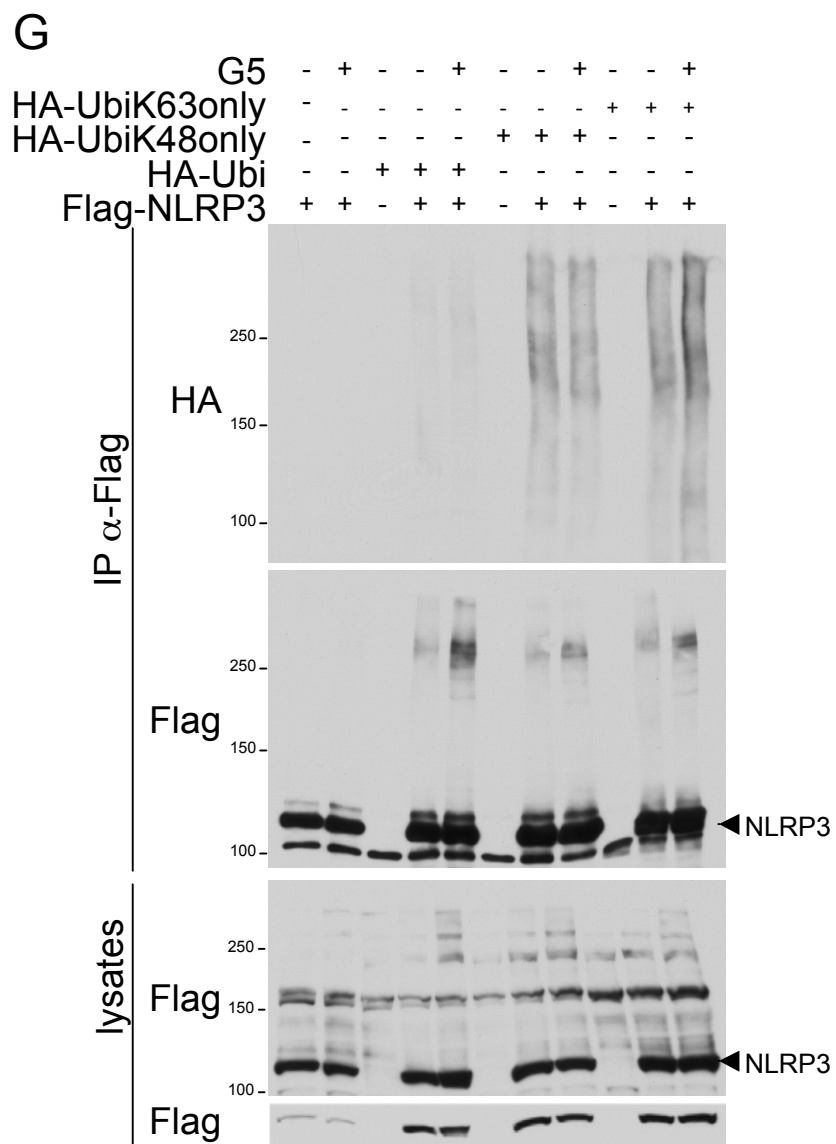


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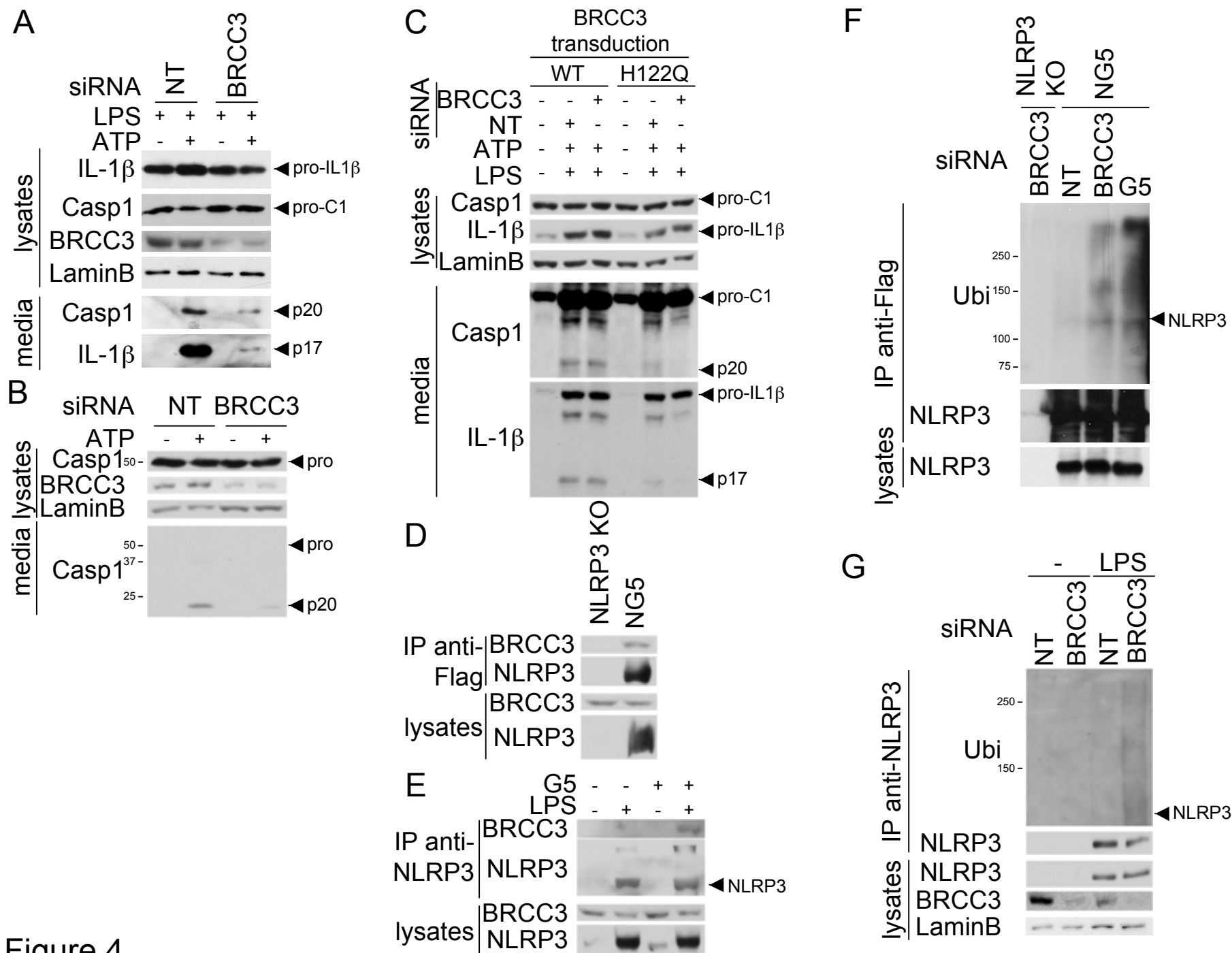


Figure 4

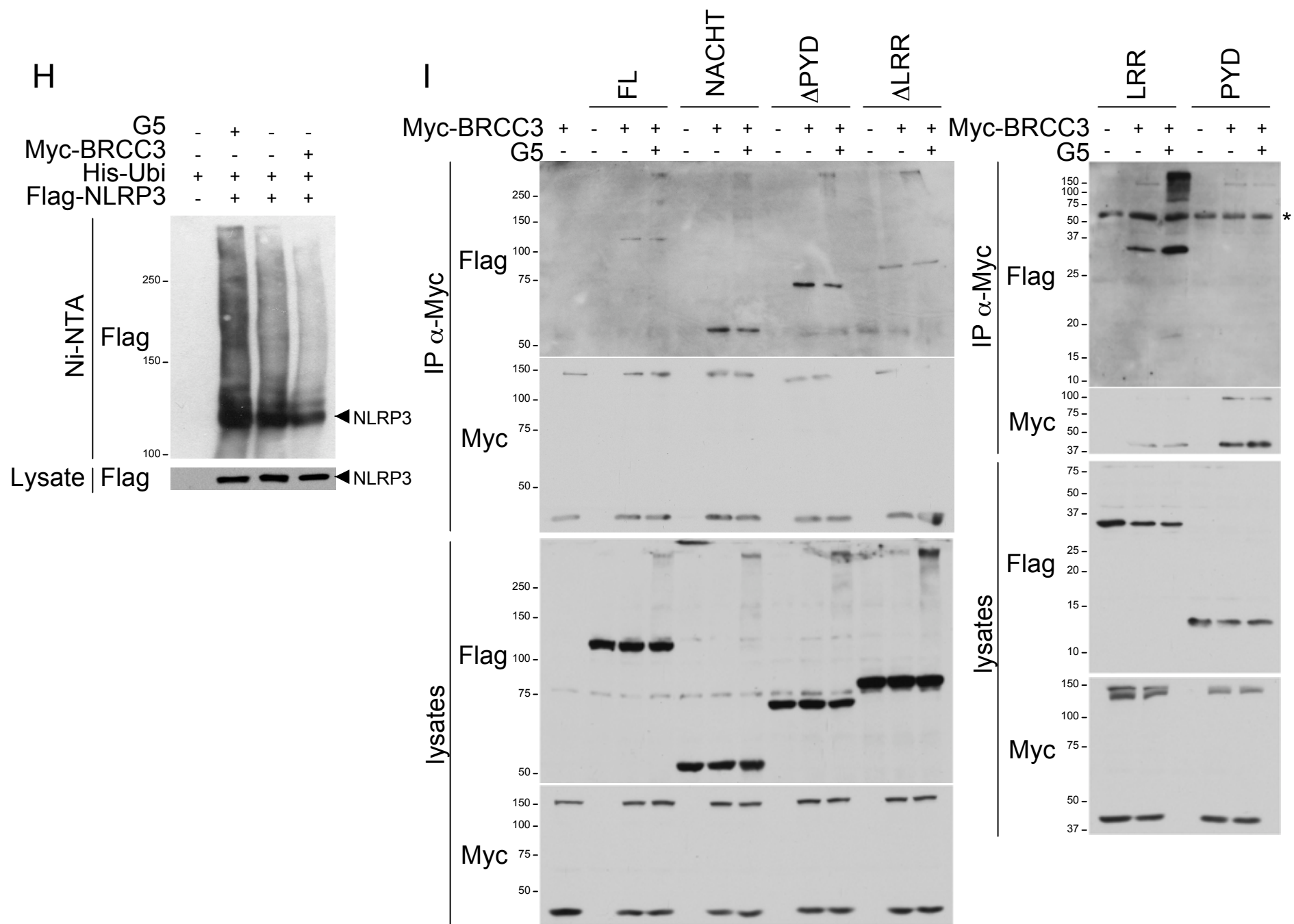


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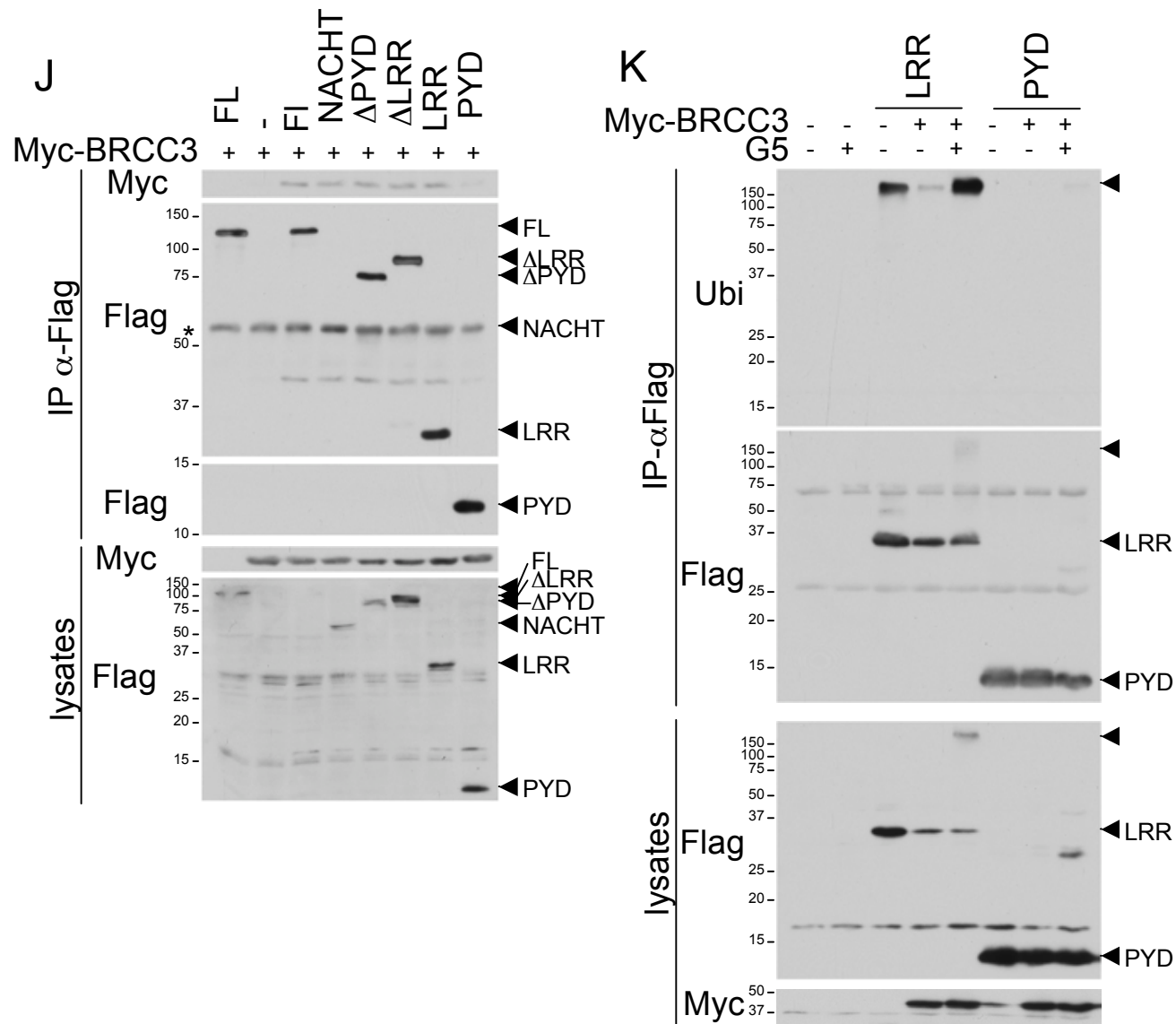


Figure 4, continued