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The NLRP3 inflammasome: activation and regulation

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Abstract

The NLRP3 inflammasome is a cytoplasmic supramolecular complex that is activated in response to cellular perturbations triggered by infection and sterile injury. Assembly of the NLRP3 inflammasome leads to the activation of caspase-1, which induces the maturation and release of interleukin-1β (IL-1β) and IL-18 as well as the cleavage of gasdermin D, which promotes a lytic form of cell death. Production of IL-1β via NLRP3 can contribute to the pathogenesis of inflammatory disease whereas aberrant IL-1β secretion through inherited NLRP3 mutations causes autoinflammatory disorders. We discuss in this review recent developments about the structure of the NLRP3 inflammasome, and the cellular processes and signaling events controlling its assembly and activation.

Keywords

Potassium efflux; Mitochondria; Golgi; Metabolism; Ubiquitination; Phosphorylation

The NLRP3 inflammasome: an essential component of the innate immune system

The innate immune system detects the presence of microbes and sterile cellular injury to initiate protective immune responses that promote the elimination of invading pathogens and the repair of damaged tissue. This initial activation of immune responses is largely mediated by host germline-encoded pattern recognition receptors (PRRs) that sense molecular motifs conserved in microbes, endogenously derived damage-associated molecules, and cellular activities induced by pathogen virulence factors [1].

One class of intracellular PRRs is the inflammasome-forming sensors that form multimeric protein complexes upon activation leading to the induction of immune responses. After

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assembly, inflammasomes enable the proteolytic activation of caspase-1 which promotes the processing and release of mature IL-1β and IL-18, as well as the cleavage of gasdermin D (GSDMD) that promotes pore formation and **pyroptosis** (see Glossary), a lytic form of cell death [2, 3]. The most well-characterized inflammasome is induced through NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3), a PRR that belongs to the **NOD-like receptor** (**NLR**) family [2, 3]. The NLRP3 inflammasome promotes the immune system to fight microbial infections; on the other hand, the aberrant activation of NLRP3 through inherited mutations causes several autoinflammatory disorders, and chronic activation of the NLRP3 inflammasome can contribute to the pathogenesis of several inflammatory disorders including diabetes, gouty arthritis, silicosis, atherosclerosis and Alzheimer's disease [4]. In addition, the NLRP3 inflammasome is important in cancer and metabolic diseases [5]. Here we focus on the recent advances regarding the structure of the NLRP3 inflammasome, and the cellular processes and signaling events controlling its assembly and activation.

NLRP3 structure

NLRP3 consists of a N-terminal pyrin domain (PYD), a centrally located adenosine triphosphatase (ATPase) domain known as nucleotide-binding oligomerization domain (NOD or NACHT) that comprises nucleotide-binding domain (NBD), helical domain 1 (HD1), winged helix domain (WHD) and helical domain 2 (HD2) and C-terminal leucine-rich repeat (LRR) domain that consists of 12 repeats (Figure 1) [6]. Like most inflammasomes, the NLRP3 inflammasome includes the adaptor ASC that is composed of a PYD that associates with the PYD of NLRP3 and a caspase activation and recruitment domain (CARD) that interacts with the CARD of caspase-1. Upon inflammasome activation, caspase-1 recruited by the oligomerized ASC can be activated through proximity-induced self-cleavage (Figure 1) [2, 3]. In mouse macrophages, NIMA-Related Kinase 7 (NEK7), a mitotic kinase that interacts with NLRP3 is required for inflammasome activation [7–9]. However, NEK7 appears to function in NLRP3 priming, but not the activation process in human macrophages [10]. In contrast, NLRP11, a NLR family member that is absent in mice, was identified as a component of the NLRP3 inflammasome required for NLRP3 activation in human macrophages [11]. Although further work is needed to verify these results, these observations suggest differential regulation of the NLRP3 inflammation across species.

The first structure of NLRP3 was revealed by cryo-electron microscopy (Cryo-EM) of PYD-deleted ADP-bound human NLRP3 in its inactive conformation in complex with an artificial dimer of NEK7 [6]. NLRP3 and NEK7 interact through two interfaces, one involving the LRRs of NLRP3 and the first half of the NEK7 C-lobe and a second interface between the NACHT domain and the second half of the NEK7 C-lobe [6]. Mutations of amino acid residues involved in the NLRP3-NEK7 interface impaired NLRP3 inflammasome activation [6]. The work suggested that in addition to the NEK7-NLRP3 interaction, further conformational changes in NLRP3 may be needed for inflammasome activation including those mediated by ATP binding and other unknown events [6]. One of these events may be the binding of the receptor for activated C kinase 1 (RACK1) with both NLRP3 and NEK7 as this interaction is important for conformational changes required for inflammasome activation [12].

Originally, it was proposed that NLRP3 exists in a monomeric state with a closed conformation causing auto-inhibition and upon stimulation, NLRP3 assumes an open conformation that enables its oligomerization and activation [3]. This concept was challenged by three recent publications revealing the Cryo-EM structure of the full-length human and mouse NLRP3 in an inactive form with and without the NLRP3 inhibitor MCC950 (also known as CRID3) [13–15]. Inactive NLRP3 forms oligomers that can assemble via LRR-LRR face-to-face interactions between two monomers, and these pairs then interact with other pairs through back-to-back LRR interactions to form a circular "double ring" or "cage" structure, while the NACHT domains barely touch each other [13–15]. The PYDs are shielded inside the "double ring" to presumably prevent inadvertent NLRP3 activation. Because the LRR-LRR face-to-face interface in inactive NLRP3 overlaps with the interface between the LRRs of NLRP3 and NEK7, the binding of NEK7 with NLRP3 could disrupt interactions that maintain the inactive state of NLRP3 (Figure 1). In the proposed activation model, NEK7 binding and other unknown events disassemble the "double ring" into a "single ring" structure and NLRP3 assumes a conformation in which the exposed PYDs are capable of recruiting and nucleating ASC, which leads to caspase-1 activation (Figure 1). There are subtle differences between the three structures of inactive NLRP3 including the number of monomers; for example, human NLRP3 exhibited a decameric structure whereas mouse NLRP3 formed 12–16-mers [13–15]. The different results may reflect technical or species-specific variability. Mutations of amino acid residues in the LRR-LRR dimerization interface of human NLRP3 led to NLRP3 auto-activation and hyperactivation, supporting a role of the LRR domain in keeping the "double ring" structure of NLRP3 inactive [13]. However, this finding contradicts two reports, in which NLRP3 lacking the LRR domain retains similar function as full-length NLRP3 [16, 17]. The reason for the discrepancy in results is unclear and warrants further investigation.

NLRP3 inflammasome activation

The NLRP3 inflammasome can be activated through three pathways: canonical, non-canonical and alternative pathways. Here we focus on canonical activation of NLRP3 and discuss briefly NLRP3 activation via the non-canonical inflammasome and the alternative pathways (Box 1). In mouse macrophages, canonical NLRP3 inflammasome activation is a two-step process. A **priming signal** is required before a subsequent signal activates the NLRP3 inflammasome. Priming is typically induced by stimulation of macrophages with **Toll-like receptor (TLR)** ligands, IL-1β or TNFα which activates NF-κB, leading to the upregulation of NLRP3 and IL-1β [18, 19]. Priming also results in post-translational modification of NLRP3, which will be discussed later, to license the activation of NLRP3. Following priming, the NLRP3 inflammasome can be activated by a wide array of stimuli including ATP, nigericin, gramicidin, bacterial toxins, and particulate matter such as crystals of silica, aluminium hydroxide, monosodium urate, and calcium pyrophosphate dihydrate. The chemical and structural diversity of these activating stimuli suggests that NLRP3 senses a common cellular signal induced by the various stimuli (Figure 2). Here we concentrate on recent progress in understanding the cellular and signaling events leading to canonical NLRP3 activation.

Activation and regulation by ions

Potassium—Potassium (K^+) efflux is induced by practically all NLRP3-activating stimuli and is required for NLRP3 activation (Figure 2) [20]. However, certain imidazoquinolinamines including imiquimod (IMQ) and CL097, and peptidoglycan can activate NLRP3 independently of K^+ efflux [21, 22]. In human monocytes activation of NLRP3 with lipopolysaccharide (LPS) alone via an alternative pathway (Box 1) is K^+ efflux independent [23]. Thus, in some cells, there are mechanisms of NLRP3 activation that can bypass K^+ efflux. In mouse bone marrow-derived macrophages, lowering the extracellular concentration of K^+ (≤ 0.5 mM) is sufficient for NLRP3 activation in the absence of additional stimulation, while increasing the extracellular K^+ concentration to 30–45 mM has an inhibitory effect on NLRP3 activation [20]. Mechanistically, bacterial toxins, the K^+ ionophores nigericin and gramicidin, extracellular ATP via the purinergic P2X7 channel, and the two-pore domain weak inwardly rectifying K^+ channel 2 (TWIK2) form pores or permeabilize the plasma membrane enabling K^+ efflux [20, 24]. Studies with a bioluminescence resonance energy transfer (BRET) assay showed that low intracellular K^+ concentration leads to a conformational change in inactive NLRP3 [25]. This structural change is promoted by a unique linker sequence and a short FISNA domain located between the PYD and the NACHT domain of NLRP3 [25]. Notably, replacement of the PYD and the linker sequence of NLRP6, another inflammasome-forming NLR family member that does not respond to K^+ efflux, with the PYD, linker sequence and FISNA domain of NLRP3 confers the ability to respond to NLRP3 stimuli [25]. However, this chimeric NLRP6 also responded to IMQ [25], a NLRP3 activator that does not induce K^+ efflux, suggesting that K^+ efflux-independent triggers induce similar conformational changes. Thus, additional studies are needed to understand how K^+ efflux triggers NLRP3 activation.

Sodium—Sodium (Na^+) influx has been suggested to regulate NLRP3 activation induced by certain agonists. Lowering extracellular Na^+ concentration suppresses NLRP3 activation induced by nigericin, gramicidin and K^+-free medium. However, replacing the extracellular Na^+ with the cation choline does not compromise NLRP3 activation mediated by ATP, silica, Al(OH)3 and aerolysin [20]. Moreover, Na^+ influx is not sufficient for NLRP3 activation because increasing cytosolic Na^+ concentrations with a Na^+ ionophore does not trigger NLRP3 activation [20]. Monosodium urate crystals increase the intracellular Na^+ concentration, resulting in water influx which reduces the intracellular K^+ concentration below the threshold required for NLRP3 activation [26]. This suggests that the regulatory role of Na^+ in NLRP3 activation is indirect and dependent on the intracellular K^+ concentration.

Chloride—The role of chloride (Cl^-) fluxes in NLRP3 activation is controversial. Chloride intracellular channels (CLICs) can function downstream of K^+ efflux to promote NLRP3-NEK7 interaction and NLRP3 activation [27]. The role of CLIC1 and CLIC4 appears complex in that these channels can also regulate NLRP3 priming including IL-1β transcription [28]. In contrast, the kinase WNK1 negatively regulates NLRP3 activation by modulating chloride/cation cotransporters and inhibiting Cl^- efflux [29]. However, chloride/cation cotransporters regulate not only intracellular Cl^-, but also intracellular K^+ [29]. Furthermore, Cl^- efflux promoted ASC oligomerization and inflammasome priming, but not

caspase-1 activation which required K^+ efflux [30]. These results suggest that Cl^- efflux alone is not sufficient for NLRP3 activation, although intracellular Cl^- may contribute to NLRP3 activation.

Calcium—Calcium (Ca^{2+}) fluxes have also been linked to NLRP3 activation. An increase in cytosolic Ca^{2+} induced in response to NLRP3 agonists was suggested to be important for NLRP3 activation [31]. A release of Ca^{2+} from the endoplasmic reticulum (ER), a major calcium reservoir, to the cytosol through the inositol 1,4,5-triphosphate receptor (IP_{3R}) was suggested to be involved because chemical or genetic inhibition of IP_{3R} prevented Ca^{2+} release and attenuated NLRP3 activation [32]. Since Ca^{2+} overload leads to mitochondrial dysfunction, it was proposed that Ca^{2+}-mediated mitochondrial damage led to NLRP3 activation [31]. However, inhibition of NLRP3 activation by chemical inhibition of IP_{3R} was independent of Ca^{2+} signaling [33]. In addition, influx of extracellular Ca^{2+} is not required for NLRP3 activation in some studies [33]. Thus, there is no convincing evidence supporting a specific role for Ca^{2+} fluxes in NLRP3 activation.

Activation and regulation by cellular organelles

Mitochondria—The role of the mitochondria in NLRP3 activation remains controversial (Figure 2). The mitochondria can provide a subcellular site for NLRP3 inflammasome assembly and molecules including mitochondrial reactive oxygen species and DNA that can promote NLRP3 activation. A role for the mitochondrial proteins mitofusin-2, cardiolipin, and mitochondrial antiviral signaling protein (MAVS) in NLRP3 activation has been proposed [34–36]. However, localization of NLRP3 to the mitochondria remains controversial [37]. Although MAVS regulates NLRP3 activation induced by RNA viruses, the role of MAVS in non-viral stimuli was not confirmed [34, 38]. Cardiolipin, a phospholipid that is normally located in the mitochondrial inner membrane and externalized in response to mitochondrial dysfunction, can bind the HD2 and the beginning of the LRR domain of NLRP3 to promote inflammasome activation [35]. However, additional studies are needed to clarify the role of cardiolipin, and mitochondrial dysfunction in general in NLRP3 activation.

Mitochondrial reactive oxygen species (mtROS) that is generated largely through the electron transport chain (ETC) complex I and III has been linked to NLRP3 activation [39]. In line with this, IMQ and CL097 were suggested to activate NLRP3 by targeting quinone oxidoreductases (NQO2) and mitochondrial Complex I which promoted mtROS production [21]. However, whether mtROS is the trigger or a consequence of NLRP3 activation was unclear. In more definitive studies, the ETC complex I or III was replaced with an alternative oxidase that maintained the normal ETC function, but is unable to generate mtROS. In the absence of mtROS, the cells exhibited normal NLRP3 activation and IL-1β release [40], suggesting that mtROS is actually not required for NLRP3 inflammasome activation.

Mitochondrial DNA (mtDNA) can be released into the cytosol from damaged mitochondria to promote NLRP3 activation. For example, oxidized mtDNA can bind and activate NLRP3 [41]. Furthermore, TLR-mediated synthesis of mtDNA was necessary for NLRP3 activation, as genetic mtDNA depletion compromised NLRP3 activation [42]. However, the release of

mtDNA can also occur as a consequence of NLRP3 activation [43]. Studies have implicated the ETC in NLRP3 activation through phosphocreatine-dependent ATP production [40]. Thus, depletion of mtDNA may indirectly impact NLRP3 activation through its effect on ETC activity. Overall, the role for mtDNA in NLRP3 activation remains controversial.

Microtubule-organizing center (MTOC)—Centrosomes, the main MTOC, are essential for organizing the microtubule network in most animal cells. There is mounting evidence that the MTOC is involved in inflammasome activation. For example, the NEK7 kinase that binds and promotes NLRP3 activation localizes to the centrosome (Figure 2) [44]. Because the centrosome regulates mitosis, it was suggested that mitosis and NLRP3 activation are mutually exclusive, due to the limited availability of NEK7 during mitosis [9]. Another centrosomal kinase, polo-like kinase 4 (PLK4), negatively regulates NLRP3 activation by phosphorylating NEK7 and inhibiting its association with NLRP3 [45]. In addition, NLRP3 localizes to the centrosomal MTOC upon activation [46, 47]. Microtubule-affinity regulating kinase 4 (MARK4) promotes the mobilization of NLRP3 to the MTOC where a single ASC inflammasome punctum assembles in each activated cell [47]. The dynein adapter histone deacetylase 6 (HDAC6) may be involved in the formation of the ASC speck via microtubule-mediated transport of inflammasome components to the MTOC [46]. However, further studies are needed to understand how NLRP3 and ASC are transported to converge into the ASC speck for inflammasome assembly and activation.

Golgi apparatus and endosomes—Mounting evidence links NLRP3 activation to the Golgi apparatus (Figure 2). In initial studies, the SCAP-SREBP2 complex associated with NLRP3 to form a ternary complex that transported NLRP3 from the ER to the Golgi apparatus for optimal inflammasome assembly [48]. More recent studies showed that NLRP3 agonists trigger the formation of dispersed trans-Golgi network (dTGN) and the recruitment of NLRP3 to phosphatidylinositol-4-phosphate (PI4P) vesicles was necessary for NLRP3 activation [37]. The recruitment of NLRP3 to the dTGN was dependent on a positively charged polybasic region located between the PYD and the NACHT domain of NLRP3 [37]. In this study, TGN38, which localizes to the Golgi, but also to membrane vesicles that recycle between the plasma membrane, endosomes and the TGN [49, 50], was used as a Golgi marker. Using different TGN markers, it was determined that upon NLRP3 stimulation the TGN remains largely intact, while TGN38 was trapped in endosomes that resemble “dispersed TGN” [51, 52]. Furthermore, retrograde vesicular trafficking of endosomes to the TGN was disrupted by NLRP3 agonists, leading to the accumulation of TGN38 and PI4P-membranes in endosomes. Genetic disruption of endosome-TGN retrograde transport induced NLRP3 activation with LPS alone [52]. Thus, this work suggested that NLRP3 senses the disruption of retrograde trafficking. However, NLRP3 stimuli can cause damage to the mitochondria and other intracellular organelles through ion fluxes which may be independent of NLRP3 activation [20]. Thus, further investigation is needed to clarify the role of the dTGN and vesicular trafficking in NLRP3 activation.

Lysosomes—The NLRP3 inflammasome is also activated by phagocytosis and accumulation of particulate matter in lysosomes, which causes membrane damage and the release of lysosomal cathepsin into the cytosol (Figure 2) [53]. Lysosomal membrane

rupture appears to be sufficient for inflammasome activation because the lysosomotropic agent Leu-Leu-O-methyl ester activates NLRP3 [20, 53]. Cathepsin inhibitors blocked caspase-1 activation induced by particulate matter [53]. However, genetic ablation of several cathepsins shows no or minimal alteration of NLRP3 activation [54]. Furthermore, particulate matter-induced cell death is independent of inflammasome activation as it proceeds in the absence of caspase-1 and gasdermin D [55]. K^+ efflux is important in inflammasome activation because K^+ flux is induced after particulate matter internalization which precedes NLRP3 activation [20]. However, the molecular mechanism that accounts for K^+ efflux after lysosomal damage remains unclear.

Other organelles—Cholesterol transport from lysosomes to the ER has been suggested to be required for NLRP3 activation, cholesterol transport blockade or depletion in ER membranes compromises NLRP3 activation [56]. However, the mechanism remains unclear. Ribosomes, the cellular sites for protein synthesis, have been proposed to regulate NLRP3 activation as halting of protein translation triggers NLRP3 activation [57]. Furthermore, polysaccharide galactosaminogalactan of the fungus *Aspergillus fumigatus* binds ribosomal proteins and blocks protein translation, which results in NLRP3 activation [58]. The role of ribosomes and protein translation in NLRP3 activation is intriguing and warrants further investigation.

Activation and regulation by metabolism

Glycolysis—During inflammation, macrophages undergo metabolic reprogramming to meet new bioenergetic demands that are required for effective macrophage function. Part of this reprogramming is the rapid upregulation of aerobic glycolysis to generate ATP from glucose [59]. Several studies have linked glycolysis to the regulation of NLRP3 activation and IL-1β production. For example, ablation of hexokinase-1, a kinase that catalyzes the first step in glycolysis, impaired LPS-induced NLRP3 activation and IL-1β release [60]. During bacterial infection, hexokinase-1 binds peptidoglycan-derived N-acetylglucosamine (GlcNAc) promoting the translocation of the enzyme from the mitochondria into the cytosol and NLRP3 activation [22]. Incubation of macrophages with 2-deoxy-D-glucose, an inhibitor of glycolysis, reduced NLRP3 activation and IL-1β production [60]. Furthermore, genetic and pharmacological inhibition of pyruvate kinase isoenzyme M2 (PKM2), an enzyme that catalyzes the last step of glycolysis, compromised NLRP3 activation [61]. In contrast to these studies, blocking glycolysis by inhibiting other glycolytic enzymes such as GAPDH or alpha-enolase, was sufficient to activate the NLRP3 inflammasome [62]. The reason for these seemingly contradictory results is unclear, but it may reflect the complexity of interfering with multiple intertwining metabolic pathways in macrophages.

Tricarboxylic acid (TCA) cycle—In LPS-activated macrophages, the TCA cycle and oxidative phosphorylation (OXPHOS) are also upregulated, followed by nitric oxide-mediated disruption of the TCA cycle, leading to the accumulation of metabolites such as succinate and itaconate [63]. Succinate stabilizes the transcription factor hypoxia inducible factor-1α (HIF-1α), increasing IL-1β production [64]. Fumarate, another intermediate of the TCA cycle, inhibited nigericin-induced pyroptosis by modifying gasdermin D cysteine residues into S-(2-succinyl)-cysteine, which reduced gasdermin D processing and pore

formation on the plasma membrane [65]. LPS also induces the production of itaconate by upregulating the expression of *cis*-aconitate decarboxylase, the enzyme responsible for the decarboxylation of the TCA cycle intermediate *cis*-aconitate in macrophages [66]. Itaconate decreases the expression of IL-1β and several components of the inflammasome thereby reducing NLRP3 activation [67]. In addition, 4-octyl-itaconate (OI), an itaconate derivative, can modulate NLRP3 activation by inhibiting the interaction of NLRP3 with NEK7 through dicarboxypropylation of C548 on NLRP3 [68]. In contrast, another study suggested that itaconate confers tolerance to NLRP3 activation by acting downstream of ASC speck formation [69]. In addition to the TCA metabolites, mitochondrial ETC activity is required for NLRP3 activation through phosphocreatine-dependent ATP production (Figure 2) [40]. These studies suggest complex regulation of the NLRP3 inflammasome during metabolic reprogramming in macrophages.

Fatty acid metabolism—Fatty acid metabolism has also been implicated in NLRP3 activation. The saturated fatty acid palmitate induces NLRP3 activation [70] and inhibiting lipid synthesis by targeting mitochondrial uncoupling protein-2 (UCP2) or fatty acid synthase (FASN), compromised NLRP3 activation [71]. While lipid synthesis is required for NLRP3 activation, fatty acid oxidation seems to regulate this inflammasome. For example, genetic ablation of NADPH oxidase 4 (NOX4) was associated with reduced expression of carnitine palmitoyltransferase 1A, a key mitochondrial enzyme in the fatty acid oxidation pathway. NOX4 deficiency and reduced fatty acid oxidation resulted in impaired NLRP3 activation, but the mechanism remains poorly understood [72]. In contrast, fatty acid oxidation generates ketone bodies. One of the ketone bodies, β-hydroxybutyrate, inhibits NLRP3 activation by suppressing K^+ efflux [73].

NLRP3 inflammasome regulation by post translational modifications (PTMs)

Recent research has revealed roles for PTMs in NLRP3 priming and activation. Here we focus on ubiquitination and phosphorylation and discuss other PTMs including sumoylation and acetylation (Box 2).

Ubiquitination

Several proteins can mediate the ubiquitination of NLRP3 through the addition of ubiquitin chains to regulate inflammasome activation (Table 1). At steady state, the F-box and leucine rich repeat protein 2 (FBXL2) mediates K48-linked polyubiquitination and degradation of NLRP3 while LPS-priming activates F-box protein O3 (FBXO3) to induce the degradation of FBXL2 which enhances NLRP3 expression [74]. The E3 ubiquitin ligase membrane-associated RING finger protein 7 (MARCH7) also limits NLRP3 expression via K48-linked ubiquitination in response to Dopamine D1 receptor signaling resulting in autophagic degradation of NLRP3 [75]. The E3 ligase tripartite motif containing protein 31 (TRIM31) also promotes proteasomal degradation of NLRP3 via K48 ubiquitination [76]. In contrast, E3 ligase ring finger protein 125 (RNF125) catalyzes K63-linked ubiquitination of the LRRs of NLRP3, which recruits E3 ligase Cbl proto-oncogene B (Cbl-b), which promotes K48-linked ubiquitination and proteasomal degradation of NLRP3 [77]. Similarly, E3 ligase

β-TrCP1 promotes proteasomal degradation of NLRP3 through K27-linked ubiquitination, which can be blocked by the interaction between NLRP3 and the transcription co-activator YAP [78]. In addition to facilitating NLRP3 degradation, ubiquitination can disrupt inflammasome assembly. Cullin1 (CUL1), a component of the Skp1-Cullin1-F-box E3 ligase complex, inhibits NLRP3 activation by binding NLRP3 and promoting its K63 ubiquitination at K689, which blocks the association of NLRP3 with ASC [79]. NLRP3-activating stimuli promote the dissociation of CUL1 from NLRP3, enhancing NLRP3 inflammasome assembly [79]. Moreover, the E3 ligase Ariadne homolog 2 (ARIH2) interacts with NLRP3 to promote its ubiquitination, while its genetic ablation inhibits NLRP3 ubiquitination and promotes NLRP3 activation [80]. In contrast to the negative role of ubiquitination in NLRP3 activation, E3 ligase Pellino2 catalyzes K63 ubiquitination of NLRP3 during priming and facilitates NLRP3 activation [81]. The E2 enzyme ubc13 potentiates NLRP3 inflammasome activation by promoting K63-linked polyubiquitination of mouse NLRP3 at K565 and K687 [82]. However, ubiquitination of of human NLRP3 at K689 (K687 in the mouse) by Cullin1 inhibits NLRP3 activation [79]. The reason for this inconsistency is unclear.

In general, deubiquitination of NLRP3 promotes its activation. For example, the UAF1/USP1 deubiquitinase complex inhibits NLRP3 degradation by removing K48 polyubiquitination chains of NLRP3 [83]. BRCC3, a deubiquitinase for NLRP3, increases NLRP3 activation through the removal of ubiquitin chains from NLRP3 [84, 85]. Likewise, knockdown of two deubiquitinases, USP7 and USP47, compromises NLRP3 activation, although the mechanism is unclear [86]. Collectively, these studies suggest that in most cases ubiquitination negatively regulates NLRP3 activation by inducing NLRP3 degradation or disrupting inflammasome assembly.

Phosphorylation

Phosphorylation regulates NLRP3 activation by regulating inflammasome priming or assembly, or altering NLRP3 subcellular localization (Table 1). Through TGR5 bile acid receptor or prostaglandin E2 (PGE2) signaling, activated PKA phosphorylates NLRP3 at S295 on the NACHT domain and inhibits its ATPase activity [87, 88]. In mouse airway epithelial cells, EphA2, a tyrosine kinase receptor, phosphorylates NLRP3 at Y132 which interferes with inflammasome assembly [89]. At the steady state, AKT limits NLRP3 activation through phosphorylation of NLRP3 at S5, which inhibits PYD-PYD interactions and reduces NLRP3 ubiquitination on K496, blocking its proteasomal degradation of NLRP3 [90]. The TGF-β activated kinase 1 (TAK1) limits TNFα-induced NLRP3 activation at the resting state by inhibiting RIPK1-dependent NF-κB and ERK signaling [91]. In some cases, phosphorylation of NLRP3 promotes inflammasome activation. For example, the *E. coli* toxin CNF1 induces NLRP3 activation through P21 activated kinase 1 (Pak1)-mediated phosphorylation of NLRP3 at T659 of NLRP3 [92]. Interestingly, phosphorylation of NLRP3 at T659 is required for the interaction between NLRP3 and NEK7 [92].

Dephosphorylation of NLRP3 at S5 by Phosphatase 2A (PP2A) promotes NLRP3 activation [93]. TANK-binding kinase 1 (TBK1) and I-kappa-B kinase epsilon (IKKε) counteract the role of PP2A on S5 to limit NLRP3 activation, although these kinases may also act on other

amino acid residues that impact NLRP3 activation [94]. Similar to PP2A, other phosphatases can also promote NLRP3 activation. For example, protein tyrosine phosphatase non-receptor 22 (PTPN22) dephosphorylates NLRP3 at Y861, reducing NLRP3 autophagic degradation [95]. Phosphatase and tensin homolog (PTEN) dephosphorylates NLRP3 at Y32 in the PYD, enhancing the interaction between NLRP3 and ASC [96].

Cross-talk between phosphorylation and ubiquitination of NLRP3 has been observed. During priming, JNK1 phosphorylates NLRP3 at S194 to induce BRCC3-mediated deubiquitination and activation of NLRP3 [97]. NLRP3 is also phosphorylated at S803 via the CSNK1A1 kinase during priming while activating signals induce NLRP3 dephosphorylation at the same residue, increasing the recruitment of NEK7 to NLRP3 which promotes BRCC3-mediated deubiquitination and activation of NLRP3 [98].

Phosphorylation can also regulate the subcellular location of NLRP3. A polybasic linker between the PYD and NACHT domain is known to enable the recruitment of NLRP3 to the dTGN (see the section of Golgi apparatus and Endosomes) [37]. Bruton's tyrosine kinase (BTK) interacts with NLRP3 and ASC [99], phosphorylates NLRP3 at tyrosine residues 136, 140, 143 and 168, which reside within or adjacent to the polybasic PYD-NACHT linker region that promotes NLRP3 translocation from the intact Golgi to the dTGN and inflammasome activation [100]. These tyrosine modifications alter the net charge of the linker region, providing a plausible mechanism by which BTK promotes NLRP3 activation [100]. In addition, phosphorylation mediated by BTK occurs within the FISNA domain, which facilitates conformation changes resulting from K^+ efflux upon NLRP3 activation [25]. Thus, further studies are warranted to determine whether BTK-mediated phosphorylation links K^+ efflux to NLRP3 activation. IKKβ activity is also required to recruit NLRP3 to TGN membranes; however, the target of IKKβ in this process is unknown [101]. In response to NLRP3-activating stimuli, diacylglycerol at the Golgi is induced, recruiting protein kinase D (PKD), which phosphorylates NLRP3 at S295 and is sufficient to release NLRP3 from mitochondria-associated ER membranes and promotes the assembly of the active inflammasome [102]. However, phosphorylation of NLRP3 at S295 by PKA inhibits NLRP3 activation [87, 88]. Further studies are needed to understand how phosphorylation at the same residue of NLRP3 results in opposite functional outcomes.

Concluding remarks

A full understanding of NLRP3 activation has been hampered by the fact that NLRP3-activating stimuli often induce intracellular signals that perturb cellular homeostasis of cellular ions, organelles and metabolism in addition to NLRP3 activation. Thus, it has been challenging to determine whether signaling events or perturbations are necessary or act in parallel to NLRP3 activation. Moreover, NLRP3 activation often displays cell-type and inter-species variation, adding more complexity to the system. Recent advances including Cryo-EM structures of NLRP3, identification of the NEK7-NLRP3 interaction and the possible role of organelle homeostasis in NLRP3 activation have fueled progress in the NLRP3 inflammasome field. The recent structural studies of inactive oligomeric NLRP3 are important in that they provide a basis for rational drug development to target the inflammasome. NLRP3 has been shown to localize to several organelles and transition to

different intracellular sites during activation. The newly discovered link between NLRP3 activation, the Golgi and endosomes is exciting, but it needs further verification and analysis. So far, with few exceptions, K^+ efflux is the most established cellular signal for NLRP3 activation. Despite certain advances, the mechanism by which K^+ efflux triggers NLRP3 activation remains obscure. Further investigation in the field is still needed with the ultimate goal of identifying unified mechanisms of NLRP3 inflammasome activation (see Outstanding questions).

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Glossary

Mitochondrial DNA (mtDNA)
circular DNA found inside mitochondria rather than the nucleus.

Mitochondrial reactive oxygen species (mtROS)
highly reactive chemicals or radicals, produced from O_2 by the mitochondrial electron transport chain during oxidative phosphorylation.

NOD-like receptors (NLRs)
also known as nucleotide-binding oligomerization domain and leucine rich repeat-containing proteins, a family of intracellular pattern recognition receptors that sense microbial molecules or homeostatic cellular perturbation.

Priming signal
pre-stimulation with microbial molecules, such as lipopolysaccharide or pro-inflammatory cytokines, to induce pro-IL-1β and to facilitate NLRP3 activation.

Pyroptosis
a form of lytic cell death that is induced by caspases and associated with cytokine release.

Toll-like receptors (TLRs)
a group of membrane-bound pattern recognition receptors that bind microbial molecules, such as lipopolysaccharide produced by many microbes. Upon binding, TLRs are activated and initiate signaling pathways leading to pro-inflammatory and anti-microbial responses.

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Box 1.

Non-canonical and alternative inflammasome activation

The non-canonical inflammasome is triggered by intracellular lipopolysaccharide (LPS) that is recognized by mouse caspase-11 and human caspase-4 or caspase-5 to trigger pyroptosis via gasdermin D cleavage [103]. Activation of the non-canonical inflammasome also activates the NLRP3 inflammasome by inducing K^+ efflux [103, 104]. In the alternative NLRP3 activation pathway, stimulation with TLR ligands including LPS directly activates NLRP3 in certain animal and human cells. For example, LPS alone activates the NLRP3 inflammasome in mouse dendritic cells and mice *in vivo* independently of P2X7 stimulation [105], although the molecular pathway in the mouse remains unknown. Furthermore, stimulation of human monocytes with TLR2 or TLR4 agonists induces IL-1β secretion, but not in human macrophages or dendritic cells [106]. LPS-induced IL-1β secretion in human monocytes depends on NLRP3-ASC-caspase-1, but is independent of K^+ efflux, ASC speck formation and pyroptosis [23]. Instead, this LPS-induced alternative pathway is mediated through TLR4-TRIF-RIPK1-FADD-caspase-8 signaling upstream of NLRP3 in human monocytes [23]. Apolipoprotein C3, a protein component of very-low-density lipoprotein (VLDL), can also engage TLR2 and TLR4, triggering the release of IL-1β in human monocytes via this pathway [107]. Activation of NLRP3 by caspase-8 is also observed during viral infection. For instance, upon sensing Influenza A virus, Z-DNA binding protein 1 regulates NLRP3 activation through RIPK1-RIPK3-caspase-8 signaling [108]. However, the mechanism by which caspase-8 activates NLRP3 remains poorly understood.

Box 2.

Sumoylation and acetylation of NLRP3

Sumoylation is a process in which small ubiquitin-like modifier-1 (SUMO-1) proteins are covalently attached to lysine residues in proteins. The SUMO E3-ligase MAPL sumoylates and suppresses NLRP3 activation, while sentrin-specific protease 6 (SENP6) and SENP7 desumoylates NLRP3, promoting inflammasome activation [109]. NLRP3 sumoylation is also mediated by tripartite motif-containing protein 28 (TRIM28), another E3 SUMO ligase, which promotes NLRP3 expression by inhibiting NLRP3 ubiquitination and proteasomal degradation [110]. SUMO-conjugating enzyme UBC9 also positively regulates NLRP3 activation through sumoylation of NLRP3 at K204 while desumoylation of NLRP3 is mediated by SENP3 [111].

Acetylation is a process in which acetyl groups are transferred from acetyl donors, such as acetyl-CoA, to proteins by acetyltransferases. Acetylation mainly occurs on lysine amino acid resides. Lysine acetyltransferase 5 (KAT5) mediates acetylation of NLRP3 at K24 which promotes inflammasome assembly by facilitating the interaction between NLRP3 and ASC or NEK7 [112]. However, another study reported that acetylation of NLRP3 at K21 and K22, instead of K24, plays a major role in promoting NLRP3 activation [113]. In contrast, NLRP3 deacetylation mediated by sirtuin 2 (SIRT2) suppresses inflammasome activation and aging-associated inflammation [113]. The reason for the discrepancy between the two studies remains unclear, although all lysine residues that are proposed to be acetylated reside in the PYD domain of NLRP3, which interacts with the PYD of ASC for inflammasome assembly. (Summarized in Table 1 in the main text.)

Outstanding Questions

What is the structural organization of the active NLRP3 inflammasome?

In addition to NEK7 binding, what events are required for NLRP3 inflammasome activation?

What are the components and the mechanism of activation of the NLRP3 inflammasome in human cells and *in vivo*?

What is the main pathway leading to NLRP3 activation in response to microbial stimuli *in vivo*?

How are intracellular K^+ levels sensed to induce the activation of NLRP3?

How are NLRP3 and ASC transported to converge into the ASC speck for inflammasome assembly and activation?

What is the main intracellular organelle involved in NLRP3 activation?

What is the role of the mitochondria in NLRP3 activation?

Highlights

The NLRP3 inflammasome, a critical component of the host innate immune system, plays an important role in microbial infection, but its aberrant activation causes inherited disorders and contributes to sporadic inflammatory diseases.

At steady state, the structure of NLRP3 is oligomeric and kept in an inactive form through interactions among the C-terminal LRR domains. In response to specific stimuli, NLRP3 forms a supramolecular complex called the inflammasome that activates caspase-1 leading to the release of IL-1β and IL-18.

The NLRP3 inflammasome senses the disturbance of intracellular homeostasis induced by a wide array of stimuli that converge into K^+ efflux, which is critical for NLRP3 activation.

Localization of NLRP3 to the dispersed trans-Golgi network has been suggested to play an important role in NLRP3 activation.

Post-translational modifications regulate the NLRP3 inflammasome at both priming and activation steps.

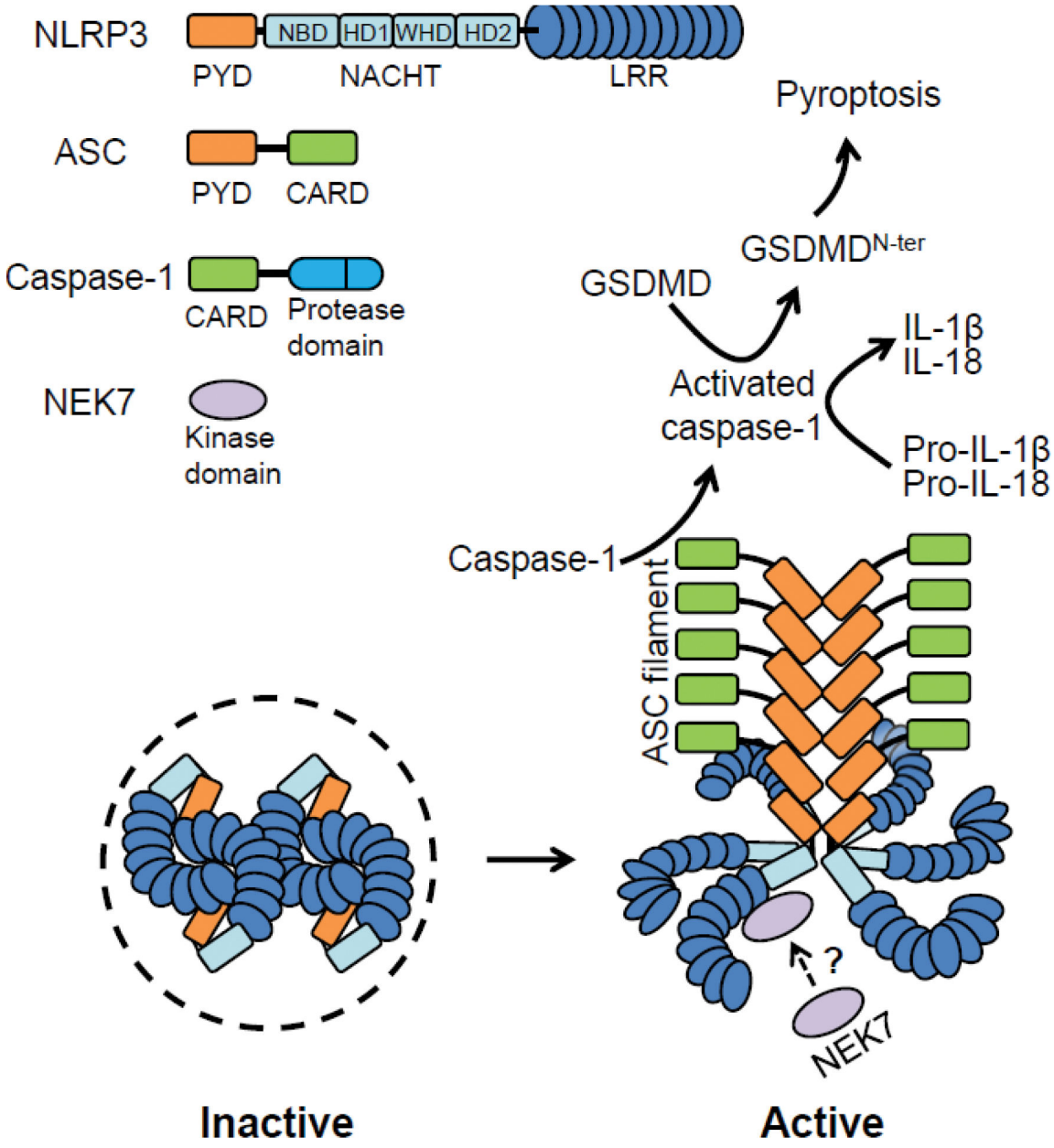

Figure 1. Components of the NLRP3 inflammasome and proposed model of NLRP3 activation.

Components of NLRP3 inflammasome: NLRP3, ASC, caspase-1 and NIMA-related kinase 7 (NEK7) (top left). The inactive form of NLRP3 (human 10-mer, mouse 12–16 mer) (bottom left). Only four of the NLRP3 monomers are displayed for simplicity to illustrate interactions among leucine-rich repeats (LRRs). The hypothetical model of the active NLRP3 inflammasome (bottom right). For simplicity, only two of the pyrin domains (PYDs) of NLRP3 interacting with the PYDs of ASC are shown. Abbreviations GSDMD, gasdermin D; IL, interleukin.

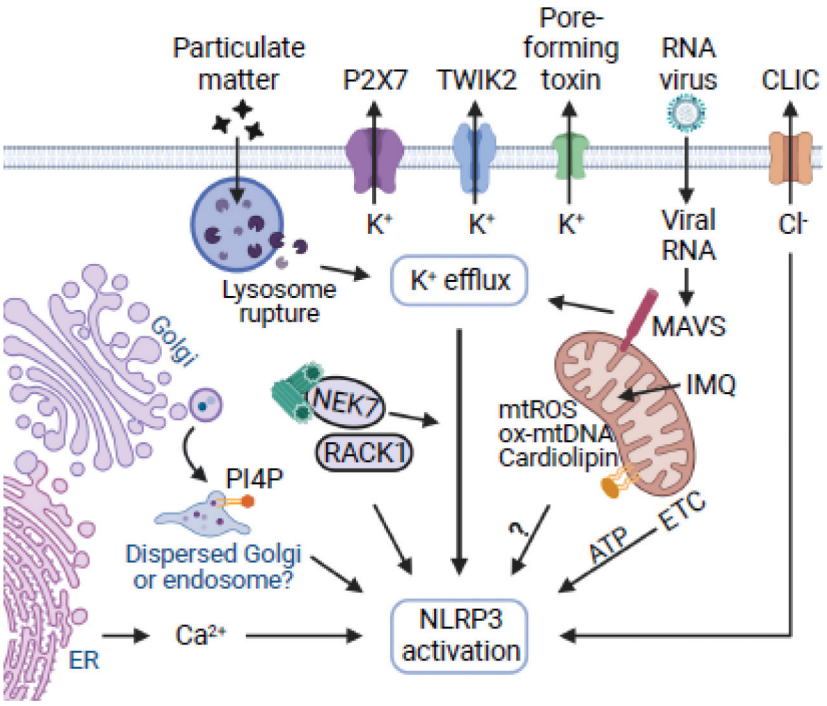

Figure 2. Cellular events leading to NLRP3 inflammasome activation.

The activation signal is provided by an array of stimuli including particulate matter, ATP, pore-forming toxins and RNA viruses. Particulate matter via lysosomal membrane damage and viral RNA through mitochondrial antiviral signaling protein (MAVS) induce K^+ efflux, which also occurs upon stimulation with most NLRP3 stimuli. As an exception, imiquimod (IMQ) activates NLRP3 without inducing K^+ efflux. NIMA-related kinase 7 (NEK7) functions downstream of K^+ efflux. NEK7 is shown associated with the centrosome (in green). The binding of receptor for activated C kinase 1 (RACK1) to NEK7 and NLRP3 is required for inflammasome activation, but the mechanism remains poorly understood. Mitochondrial ETC sustains NLRP3 activation via phosphocreatine-dependent generation of ATP. Dysfunction of the mitochondria is associated with the release of mitochondrial reactive oxygen species (mtROS) and mitochondrial DNA (mtDNA) into the cytosol as well as localization of cardiolipin to the outer mitochondrial membrane which may promote NLRP3 activation. Upon stimulation, NLRP3 transition to membranes of the dispersed trans-Golgi network (TGN) or endosomes which may be important for inflammasome activation. CLIC, chloride intracellular channel; ER, endoplasmic reticulum; ETC, electron transport chain; ox-mtDNA, oxidized; PI4P, phosphatidylinositol 4-phosphate; P2X7, purinergic receptor P2X 7; TWIK2, two-pore domain weak inwardly rectifying K^+ channel 2. This figure was created with resources from BioRender.com

Table 1.

Regulation of NLRP3 through PTMs[a]

PTM	Effect on NLRP3 activation	Enzyme	PTM site[b]	Refs
Ubiquitination	Positive	Pellino2	Unknown	[81]
		ubc13	Human: K567, K689; mouse: K565, K687	[82]
	Negative	FBXL2	Human: K689; mouse: K687	[74]
		MARCH7	Unknown	[75]
		TRIM31	Unknown	[76]
		RNF125	Unknown	[77]
		Cbl-b	Human: K496; mouse: K492	[77]
		β-TrCP1	Human: K384; mouse: K380	[78]
		Cullin1	Human: K689; mouse: K687	[79]
		ARIH2	Unknown	[80]
Deubiquitination	Positive	UAF1/USP1	Unknown	[83]
		BRCC3	Unknown	[84,85]
		USP7/USP47	Unknown	[86]
Phosphorylation	Positive	Pak1	Human: T659; mouse: T657	[92]
		JNK1	Human: S198; mouse: S194	[97]
		BTK	Human: Y136, Y140, Y143[c], Y168; mouse: Y132, Y136, Y164	[100]
		PKD	Human: S295; mouse: S291	[102]
	Negative	PKA	Human: S295; mouse: S291	[87,88]
		EphA2	Human: K136; mouse: K132	[89]
		AKT	Human: S5; mouse: S3	[90]
		CSNK1A1	Human: S806; mouse: S803	[98]
Dephosphorylation	Positive	PP2A	Human: S5; mouse: S3	[93]
		PTPN22	Human: Y861; mouse: Y858	[95]
		PTEN	Human: Y32; mouse: Y30	[96]
Sumoylation	Positive	TRIM28	Unknown	[110]
		UBC9	Human: K204; mouse: K200	[111]
	Negative	MAPL	Unknown	[109]
Desumoylation	Positive	SENP6/7	Unknown	[109]
	Negative	SENP3	Human: K204; mouse: K200	[111]
Acetylation	Positive	KAT5	Human: K26; mouse: K24	[112]
Deacetylation	Negative	SIRT2	Human: K23, K24; mouse: K21, K22	[113]

[a]ARIH2, ariadne homolog 2; BRCC3, BRCA1/BRCA2-Containing Complex Subunit 3; BTK, Bruton's tyrosine kinase; Cbl-b, Cbl proto-oncogene B; CSNK1A1, Casein Kinase 1 Alpha 1; EphA2, Eph receptor A2; FBXL2, F-box and leucine rich repeat protein 2; JNK1, c-Jun N-terminal kinase 1; KAT5, lysine acetyltransferase 5; MAPL, mitochondrial-anchored protein ligase; MARCH7, membrane-associated RING finger protein 7; Pak1, p21-activated kinase 1; PKA, protein kinase A; PKD, protein kinase D; PP2A, protein phosphatase 2; PTEN, phosphatase and tensin homolog; PTM, post-translational modification; PTPN22, protein tyrosine phosphatase non-receptor type 22; RNF125, ring finger protein 125; SENP3, sentrin-specific protease 3; SIRT2, Sirtuin 2; β-TrCP1, β-transducin repeat containing E3 ubiquitin protein ligase; TRIM31, tripartite

motif containing protein 31; UAF1, Ubiquitin specific peptidase 1-associated factor 1; Ubc13, ubiquitin conjugating enzyme 13; USP1, Ubiquitin Specific Peptidase 1

[b]The numbering of amino acid residues is based on NP_004886.3 for *Homo sapiens,* and NP_665826.1 for *Mus musculus.*

[c]Y143 of human NLRP3 is not conserved in the mouse.