Effects of Phosphorylation on the NLRP3 Inflammasome

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Abstract

The pyrin domain containing Nod-like receptors (NLRPs) are a family of pattern recognition receptors known to regulate an array of immune signaling pathways. Emergent studies demonstrate the potential for regulatory control of inflammasome assembly by phosphorylation, notably NLRP3. Over a dozen phosphorylation sites have been identified for NLRP3 with many more suggested by phosphoproteomic studies of the NLRP family. Well characterized NLRP3 phosphorylation events include Ser198 by c-Jun terminal kinase (JNK), Ser295 by protein kinase D (PKD) and/or protein kinase A (PKA), and Tyr861 by an unknown kinase but is dephosphorylated by protein tyrosine phosphatase non-receptor 22 (PTPN22). Since the PKA- and PKD-dependent phosphorylation of NLRP3 at Ser295 is best characterized, we provide detailed review of this aspect of NLRP3 regulation. Phosphorylation of Ser295 can attenuate ATPase activity as compared to its dephosphorylated counterpart, and this event is likely unique to NLRP3. *In silico* modeling of NLRP3 is useful in predicting how Ser295 phosphorylation might impact upon the structural topology of the ATP-binding domain to influence catalytic activity. It is important to gain as complete understanding as possible of the complex phosphorylation-mediated mechanisms of regulation for NLRP3 in part because of its involvement in many pathological processes.

1. Introduction

1.1 Pattern recognition receptors in innate immunity

Vertebrates rely on two highly conserved and specialized defense strategies: the innate and adaptive immune systems. The innate immune system functions as the first line of defense in reconciling injury and infection through the early detection of impending threats, and consequential triggering of proinflammatory responses[1]. These recognition and response mechanisms are mediated by the pattern recognition receptors (PRRs) during both microbial infection and anomalous endogenous signaling, including the aberrant localization of danger signals, the formation of abnormal molecular complexes and other indicators of cellular stress[2]. Evolutionarily conserved microbial motifs recognized by PRRs are referred to as pathogen-associated molecular patterns (PAMPs) and irregular host-derived signals as danger-associated molecular patterns (DAMPs). PRRs are expressed primarily in immune and inflammatory cells such as monocytes, macrophages, neutrophils, epithelial and dendritic cells[3].

Members of the PRR superfamily are broadly localized, functioning in the extracellular milieu, plasma membrane, endosomal compartments and the cytosol. Five classes of PRR families have been identified: the membrane-bound Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), the cytoplasmic retinoic acid-inducible gene-I-like receptors (RLRs), nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) and the absent in melanoma 2-like receptors (ALRs)[4]. The comparable structural domain composition of the PRR families reflect their collective role in the recognition of danger signals and the subsequent activation of pro-inflammatory signal transduction pathways. These pathways often converge on the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or antiviral type I interferon (IFN) pathways, or in the case of NLRs on the maturation and secretion of pro-inflammatory cytokines[5].

1.2 NLRs Nucleate Signaling Complexes in Response to PAMPs or DAMPs

Over the past decade, certain PRRs, including the NLRs and ALRs, have emerged as key sensors of intracellular danger signals. These receptors exhibit the capacity to nucleate multimeric signaling complexes, termed inflammasomes, in response to diverse stimuli[6]. The first NLR inflammasome (NLRP3) was characterized as a caspase-1-activating complex in which proximity-induced auto-proteolysis activated the protease to cleave and maturate the pro-inflammatory interleukin (IL)-1β precursor, pro-IL-1β[7]. The majority of the inflammasome forming sensors described to date are NLR receptors[8]. The NLR protein family is comprised of 22 members, characterized by a central nucleotide-binding domain (NACHT - [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (in-compatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)]), and discernable by their N-terminal effector domains[9]. The family can be subdivided into the NLRA, NLRB, NLRC or NLRP subfamilies based on whether the N-terminus contains an acidic domain, Baculovirus IAP repeat (BIR), caspase recruitment domain (CARD), or pyrin domain (PYD), respectively[10]. NLRP1, NLRP3 and NLRC4 constitute the best-characterized inflammasomes[11]; however, evidence also supports the existence of inflammasome complexes nucleated by NLRP6[12], NLRP7[13], NLRP12[14] and AIM2[15,16]. Although the potential inflammasome assembly of the remaining NLRP proteins is largely uncharacterized, rapidly developing models suggest these proteins play a role of paramount importance in initiating and directing the inflammatory response to cellular injury[17]. Regrettably, critical aspects pertaining to the underlying biology of this family and their signaling complexes remain to be elucidated.

1.2 NLRPs are STAND ATPase family members

Comparative sequence analyses of the 14 NLRP genes reveal conserved ATP-binding motifs within the central NACHT domain that have designated them as members of the Signal Transduction ATPases with Numerous Domains (STAND) clade, related to the larger ATPases-Associated with various cellular Activities (AAA+ ATPase) superfamily of proteins[18]. The majority of STAND P-Loop ATPases are modular proteins, containing three or more domains involved in DNA or protein binding, signal transduction and scaffolding[19]. Structural data and biochemical investigations of several family members have revealed a common mechanism of regulation. STAND proteins have a conserved core containing ATPase activity, as well as key effector domains involved in sensing of stimuli and in downstream signaling[19]. A conserved mechanism of activation has been proposed, based on the structural data of four STAND ATPases[20]. Briefly, the integrated data suggest that these ATPases function as regulated molecular switches, which undergo structural reorganizations corresponding to monomeric, ADP-bound forms associated with the "off position", and oligomeric, ATP-bound forms associated with the "on position", the latter of which can initiate downstream signal transduction pathways via protein-protein interactions mediated by the effector domains.

To date, only NLRP3[21] ,NLRP7[22] and NLRP12[23] have been empirically demonstrated to possess ATP-binding potential and intrinsic ATPase activity. While it seems evident that NLRP1 binds ATP[24], the capacity of NLRP1 to hydrolyze ATP is not yet clear. Although the seminal paper linked the contribution of nucleotide-binding to the functional role of NLRP1, a soluble fragment of the protein containing the NACHT domain and the LRR exhibited negligible ability to hydrolyze triphosphate nucleotides[25]. The basic biochemistry of these proteins remains elusive and under-characterized, so further description of the enzymology and the role of ATP in driving inflammasome activation and the ensuing inflammatory signaling pathways will be critical for a comprehensive understanding of this family and their role in human disease.

1.3 Conserved Structural Motifs Present in the NLRP-NACHT Domain

Several distinct motifs cooperate to enable nucleotide-binding and/or hydrolysis. These motifs, including their functional importance and conservation among the various NLRP family members, have been previously reviewed in detail[20,26,9], but the specific function of each in the context of NLRP3 is briefly summarized here.

The *Walker A motif*, containing the consensus sequence GXXGXGK(S/T), forms an integral P loop that is highly conserved across the entire family of STAND ATPases, including a conserved Lys residue (Lys232 in NLRP3) that provides a direct stabilizing interaction with the terminal γ-phosphate

of the nucleotide. Mutation of Gly231, Lys232 and Thr233 to Ala residues was associated with reduced ATP binding and disrupted NLRP3 inflammasome-dependent signaling, including a reduction in caspase-1 activation, IL-1β maturation, cell death, macromolecular complex formation, self-association, and association with the inflammasome component ASC, all indicating the importance of ATP-binding in NLRP3 function[21,27].

The *Walker B motif* is characterized by the consensus sequence DGX(D/E)E and contains conserved acidic residues that coordinate Mg^{2+} ion binding and aid in the priming of an H_2O molecule thought to be important in the hydrolysis of ATP (Asp302, Asp305 and Glu306 in NLRP3). However, mutations of this motif in NLRP12 did not abolish nucleotide hydrolysis[23], suggesting that ATP-binding and hydrolysis is driven primarily by other key nucleotide-binding regions of NLRPs. Homology modeling suggests that key regions of the NLRP3 Walker B site are not located in close proximity to the predicted nucleotide-binding site, which could clarify the lack of global influence of Walker B mutations on function.

The *Arginine finger motif* is characterized by the conserved CRE sequence. The positive charge of the aspartic acid residue acts to stabilize the negatively charged phosphate moieties present in ATP. The net effect is to render the γ-phosphate a better electrophile, thereby enabling the effective hydrolysis of ATP. In most AAA+ ATPases, this arginine residue is contributed in trans from a neighboring subunit present in a homo-oligomeric complex[28].

The *Sensor 1 motif* is comprised of three hydrophobic residues followed by two Ser/Thr residues and a conserved Arg (Leu346 to Arg351 in NLRP3), all of which contribute to intramolecular interactions with elements of the Walker A and B motifs in the NACHT domain in addition to binding the nucleotide.

The *Sensor 2 motif* is the final conserved functional element of the NACHT domain. It is characterized by a conserved Arg or Lys residue (Arg366 in NLRP3) that also assists in coordinating nucleotide-binding, hydrolysis, and coordinating global conformational changes between subunits.

1.4 NLR Phosphorylation is an Emerging Regulatory Mechanism

In addition to transcriptional priming, which has been meticulously reviewed[29,30,31], post-translational modifications of inflammasome components have emerged as key checkpoints in the highly-regulated processes of inflammasome activation. These broadly regulated mechanisms shape distinct biochemical responses and have been shown to alter NLRP3 protein-protein interactions, catalytic activity, sub-cellular localization, NLRP3 nucleation, and thus activation status and inflammatory signaling[32]. These modifications include ubiquitination, alkylation, S-nitrosylation,

proteolytic processing, ADP-ribosylation, and phosphorylation[33]. Protein phosphorylation is the most common mechanism in the regulation of enzymatic output and functionality; indeed, it has been speculated that one-third of all proteins in the human proteome are substrates for phosphorylation[34]. Among other processes, phosphorylation can transiently alter the intrinsic biological activity, structural conformation, subcellular localization, half-life and protein-protein docking interactions of the enzyme[35]. Recent evidence supports a significant role for phosphorylation events in both the positive and negative regulation of various NLR inflammasomes (Table 1). Indeed, evidence is quickly amassing to suggest that protein phosphorylation events are pervasive in inflammasome regulation across the NLR family, as well as in concomitant signaling pathways (reviewed in [36]). While many phosphorylation events have been characterized (with both high throughput and low throughput methods), it is clear that we do not yet have a full appreciation of how these important regulatory steps work synergistically to regulate inflammation. Various kinases and phosphatases have been identified to associate with different NLRs, yet not all phosphorylation events have been linked with an effector kinase/phosphatase system. In addition, not all phosphorylation events have been linked with clear functional effects on inflammasome regulation. Thorough biochemical assessments are still required to comprehensively identify all phosphorylation events and to assess the functional implications on NLR enzymology.

2. Phosphorylation and Regulation of the NLRP3 Inflammasome

NLRP3 is the best studied NLRP protein, and is considered to be the prototypical inflammasome-forming member of the family. In the canonical activation pathway, two distinct and critical steps are essential for assembly of the NLRP3 inflammasome: an initial transcriptional priming step, and a second, oligomerization and assembly step[37] (Figure 1). Each stage is meticulously controlled and has been extensively profiled in several publications[38,39,40,41]. As a result, this review will detail the recent advances made regarding phosphorylation events and their regulation of NLRP3 inflammasome signaling, with an additional specific focus on cAMP-dependent protein kinase (PKA)-dependent phosphorylation of NLRP3.

2.1 NLRP3 Phosphorylation Events

Current research into NLRP3 phosphorylation has revealed 13 unique sites (Table 2), reported as outputs of both high throughput methods (i.e., identification of sites using only discovery mass spectrometry) and low throughput methods (i.e., sites were identified and then validated with other biochemical methods). These NLRP3 phosphorylation events have been linked to the induction or

inhibition of inflammasome activity (Table 2), changes in NLRP3 protein conformation, the regulation of other molecular associations, as well as to the occurrence of other post-translational modifications (such as ubiquitination).

In 2016, a mechanism of NLRP3 inflammasome inhibition by tyrosine phosphorylation of NLRP3 at Tyr861 was reported by Spalinger and colleagues[42]. Loss of the protein tyrosine phosphatase non-receptor 22 (PTPN22) was associated with augmented phosphorylation at Tyr861, and decreased caspase-1 activation and IL-1β maturation. Phosphomimetic mutant NLRP3-Tyr861Glu attenuated inflammasome activity, while the NLRP3-Tyr861Phe mutant resulted in enhanced NLRP3 activation. The investigators also showed that PTPN22 could directly interact with NLRP3 in the presence of ASC.

Six phosphorylation sites (Tyr13, Ser163, Ser198, Ser334, Ser728 and Ser975) were identified in 2017 using a HEK293T reconstitution cell system in which FLAG-tagged NLRP3, ASC, pro-caspase-1 and pro-IL-1β were expressed. The NLRP3 inflammasome was captured by immunoprecipitation during priming and analyzed with discovery mass spectrometry (LC-MS/MS)[43]. From this survey, only Ser198 (Ser194 in mouse) was found to have any demonstrable impact on inflammasome activation and was subjected to further interrogation. Through site-directed alanine replacement scanning, the investigators found that only NLRP3-Ser198Ala exhibited an obvious impact on inflammasome signaling, as evidenced by decreased pro-IL-1β maturation. Subsequent expression of NLRP3-Ser198Ala in immortalized human bone marrow-derived macrophages or knock-in mice harboring the *Nlrp3* Ser194Ala allele (*Nlrp3*$^{S194A/S194A}$) provided confirmation of results. The phosphorylation event had a positive priming effect on NLRP3 that was essential for inflammasome activation. Interestingly, the phosphorylation of S194 was only observed after priming and was not induced by PAMP or DAMP stimulation of unprimed cells. Further kinase profiling and analyses with selective small molecule inhibitors revealed that c-Jun terminal kinase 1 (JNK1) could directly phosphorylate NLRP3 at Ser194 which in turn facilitated the self-association and activation of the NLRP3 inflammasome. Ultimately, the phosphorylation of Ser194 by JNK1 was shown to play a critical role in deubiquitination of NLRP3 during priming, an effect linked to the molecular interaction of NLRP3 with BRCC3 (a Lys63-specific deubiquitinase). Inactive, monomeric NLRP3 is maintained in a ubiquitinated state, thereby impeding oligomerization and inflammasome activation until a priming signal activates the BRCC3 ubiquitinase. This Zn^{2+}-dependent metalloprotease promotes the deubiquitination of the NLRP3-LRR domain[44]. Recent studies have suggested that the deubiquitination of NLRP3 is indispensable for the activation of the inflammasome, as pan-inhibition of deubiquitinases can block NLRP3 activation[45].

In another 2017 study, Zhang and colleagues demonstrated a link between protein kinase D (PKD) and NLRP3 inflammasome activation[46]. The investigators established diacylglycerol (DAG) and PKD enrichment in the Golgi fraction in response to NLRP3 inflammasome activation by various DAMPs/PAMPs. Subsequent investigations with PKD knockouts and small molecule inhibitors corroborated the requirement of PKD activity for effective inflammasome activation, potentially by mediating the recruitment of ASC to NLRP3. Through mutational analysis and the use of an antibody with immunoreactivity toward PKD consensus sites (i.e., [I/L]-X-[R]-X-[S/T]), a phosphorylation site was identified within the NACHT domain of mouse NLRP3 at murine Ser291 (corresponding to Ser295 in human NLRP3). We note an apparent discrepancy in the numbering of residues in the Zhang *et al*. publication, the authors designate the phosphorylated serine in the mouse NLRP3 protein as Ser293; however, in verifying the surrounding sequence (displayed in Figure S4D of the Zhang *et al.* 2017 publication) against UniProtKB Q8R4B8-1, we determined that the authors were referring to sequence corresponding to residues 281-303, not 283-308. We also verified that no other murine NLRP3 isoforms listed in the UniProtKB or NCBI databases are numbered as listed in the Zhang publication. Consequently, the following descriptions will reference PKD-dependent phosphorylation of Ser295 (human) and Ser291 (mouse). The phosphorylation site was confirmed with mass spectrometry and the use of a phosphospecific anti-[pSer291]-NLRP3 antibody. The authors also revealed that Ser295 phosphorylation was detected in the mature inflammasome downstream of NLRP3 self-oligomerization and suggested that the PKD-mediated phosphorylation could release NLRP3 from mitochondria-associated ER membranes, allowing for the assembly of a mature inflammasome. Furthermore, the oligomerization of a phosphomimetic NLRP3 (i.e., Ser291Glu) was abolished in a reconstitution system, whereas the oligomerization of either Ser291Ala or wild-type NLRP3 was unaffected.

Also in 2017, Stutz and colleagues independently mapped additional NLRP3 phosphorylation events using quantitative proteomics[47]. The phosphorylation of Ser5, Ser161 and Ser728 residues was identified following overexpression of FLAG-NLRP3 in a murine immortalized macrophage cell line and stimulation with various DAMPs/PAMPs. Significantly, the phosphorylation of the Ser5 site within the PYD was linked to inflammasome inhibition. In this regard, structural modeling suggested that Ser5 phosphorylation could neutralize a charged helical surface that acts as the interface for homotypic PYD-PYD interactions between NLRP3 molecules as well as heterotypic interactions between NLRP3 and ASC.

Three additional NLRP3 phosphorylation events (Ser233, Ser387, Ser436) were annotated as a result of other high-throughput discovery mass spectrometry studies[48,49,50]. These phosphorylations have yet to be validated, and it is unknown if they are linked to any regulatory roles in priming or assembly.

Finally, two reports published in 2016 described the actions of cAMP-dependent protein kinase (PKA) toward NLRP3[51,52]. The impact of PKA phosphorylation on NLRP3 is reviewed in detail in Sections 3 and 4 of this review. Briefly, PKA was found to phosphorylate human NLRP3 on Ser295 (murine residue Ser291) in the NACHT domain, resulting in an inhibition of inflammasome signaling.

2.2 Protein Phosphatases and the Dephosphorylation of NLRP3

The protein kinase catalyzed addition of a phosphoryl group to a substrate protein is a reversible modification that is subsequently removed by the hydrolytic activity of protein phosphatases[53]. Thus, confirming, characterizing and identifying the mechanisms of action of NLRP3-associated phosphatase holoenzymes will provide an essential understanding of NLRP3 functionality and inflammasome regulation. To date, a limited number of phosphatases have been identified to drive NLRP3 dephosphorylation under certain contexts. The afore mentioned protein tyrosine phosphatase PTPN22 is responsible for dephosphorylation of the pTyr861 residue[42]. The pSer5 residue, which inhibits inflammasome activation during priming[47], is suggested to be the target of protein phosphatase type-2A (PP2A). Pretreatment with okadaic acid, a PP2A and PP2A-like phosphatase inhibitor, resulted in increased Ser5 phosphorylation of NLRP3 in primed macrophages. These results were substantiated with knockdown of the PP2A catalytic subunit (PPP2AC). As with other fields of investigation, the definition of the cognate protein phosphatases responsible for dephosphorylation of specific NLRP sites has lagged behind the discovery of the phosphorylation events driven by the protein kinases. While investigations have advanced understanding of phosphatase involvement, (e.g., by linking PPP2AC and PTPN22 to the Ser5 and Tyr861 residues of NLRP3, respectively), additional focused studies are still required. As an example, it is not yet known how the regulatory/targeting subunits of the PP2A holoenzyme complex integrate with NLRP3 to control inflammasome activity. Furthermore, studies have yet to define the protein phosphatase responsible for maintaining Ser295 in the dephosphorylated state. Thus, identifying and characterizing the mechanism of action of the specific NLRP3-associated phosphatase holoenzyme(s) will add essential understanding to NLRP3 functionality and inflammasome regulation. Moreover, the comprehensive examination of the specific phosphatases involved in the regulation of all the various NLRPs will be a worthy research pursuit.

3. PKA-dependent phosphorylation and the regulation of NLRP inflammasomes

3.1 Regulation of the NLRP3 Inflammasome by cAMP

A link between cyclic 3',5'-adenosine monophosphate (cAMP) and NLRP3 inflammasome activity has been observed by multiple groups. To date, four mechanisms of cAMP-dependent regulation have been studied (Figure 2A): (1) prostaglandin E_2 (PGE_2) signaling via the PGE_2 receptors EP4 and EP2[51], (2) bile acid signaling via the transmembrane G-coupled receptor-5 (TGR5)[52], dopamine binding to the dopamine D1 receptor (DRD1)[54], and extracellular Ca^{2+} ions signaling via the G protein-coupled calcium-sensing receptor (CASR)[55]. In each case, activation of the receptor influences the activity of adenylyl cyclase (ADCY), which catalyzes the conversion of ATP to cAMP. In some cases, the receptors may stimulate the phospholipase C (PLC)-dependent cleavage of phospholipid membrane component phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and DAG. IP_3 interaction with its receptor (IP_3R) on the endoplasmic reticulum induces Ca^{2+} ion release and an increase in cytoplasmic concentration. Increased cytosolic Ca^{2+} concentration is a known activating stimulus of the NLRP3 inflammasome, while enhanced cAMP levels drove NLRP3 inhibition. Additionally, Yan and colleagues suggested that direct cAMP binding (as demonstrated by co-IP) could trigger K48 linked ubiquitination by the Membrane Associated Ring-CH-Type Finger 7 (MARCH7) E3 ubiquitin ligase, driving NLRP3 ubiquitination and autophagy mediated NLRP3 degradation[54]. These studies highlight the complexity of cAMP-dependent modulation of inflammasome activity, and offer a possible explanation for the wide breadth of NLRP activating ligands.

3.2 Identification of PKA-dependent phosphorylation of NLRP3

Two recent studies provide clear evidence that NLRP3 is a direct target of cAMP-dependent protein kinase (PKA). In this regard, the kinase was demonstrated to phosphorylate human NLRP3 at Ser295 (murine residue Ser291) in the NACHT domain and provide inhibition of NLRP3-dependent inflammasome signaling. This phosphorylation was in response to cellular exposures that activate adenylyl cyclase to elevate intracellular [cAMP] and trigger the subsequent activation of PKA, namely prostaglandin E2 signaling via the E-prostanoid (EP)-4 receptor[51] and bile acid signaling via the transmembrane G protein-coupled receptor (TGR)-5[52].

Through mutational analysis and the use of an antibody with immunoreactivity toward phosphorylated PKA consensus sites (i.e., [R/K](2)-X-[S/T]), Mortimer and colleagues revealed PKA-dependent phosphorylation of NLRP3 at Ser295 that resulted in the rapid inhibition of NLRP3 inflammasome activity[51]. The stimulation of PKA signaling prior to and following activation of the inflammasome could abrogate pro-caspase-1 processing and IL-1β maturation of murine bone marrow-derived macrophages (BMDMs) and human PMA-differentiated THP-1 cells. Moreover, PKA itself was

associated with NLRP3 prior to inflammasome activation as demonstrated with co-immunoprecipitation. The phosphorylation of recombinant GST-NLRP3 protein with PKA *in vitro* could suppress the enzymatic ATPase activity of NLRP3 when compared to its unphosphorylated counterpart. Furthermore, the overexpression of an NLRP3-Ser295Ala mutant in HEK293T cells phenocopied human cryopyrin-associated periodic syndrome (CAPS) mutants associated with unrestrained NLRP3 inflammasome activation.

Likewise, Guo and colleagues independently reported that PKA-induced phosphorylation of NLRP3 could serve as a brake on inflammasome activation[52]. This group demonstrated the accumulation of NLRP3 phosphorylation (with an antibody reactive against phosphorylated PKA substrate sites) upon cAMP activation with forskolin treatment of macrophages. The investigators also independently confirmed that the PKA catalytic subunit could associate with NLRP3, and refined the interaction location to the NACHT and LRR regions of the protein. Truncation mutants were used to localize the PKA-dependent phosphorylation to the NACHT domain and then mutational alanine scanning identified Ser291 (in the murine protein) to be the principal site for phosphorylation by PKA in a HEK293T overexpression system. Finally, the site of phosphorylation was empirically confirmed with mass spectrometry. The investigators confirmed that this mechanism of inhibition may contribute to disease, the PKA-induced phosphorylation of NLRP3 resulted in decreased inflammatory responses *in vivo*, blocking NLRP3 inflammasome-dependent LPS-induced systemic inflammation, alum-induced peritoneal inflammation and type-2 diabetes-related inflammation.

In summary, both studies demonstrate that PKA interacted directly with NLRP3 and that PKA driven phosphorylation of NLRP3 at Ser295 (or Ser291 in the mouse) prevented inflammasome formation, and resulted in the disassembly of active inflammasome complexes, abrogating the downstream cleavage of caspase-1 and pro-IL-1β. The findings of Guo *et al*. expanded upon the mechanism of inhibition by identifying that phosphorylation of murine NLRP3 at Ser291 was also associated with altered ubiquitinylation on the NACHT and LRR domains (Figure 2A). The expression of a NLRP3-S291A mutant in HEK293T cells did not accumulate Lys48- or Lys63-linked polyubiquitin chains to the same degree as the wild-type NLRP3 when phosphorylated by PKA. While these studies offer reasonable evidence to support a major role for PKA in the direct regulation of NLRP3 inflammasome, several matters remain unexplored. These include the phosphorylation status of the Ser295 residue under basal conditions, and the precise mechanism of action whereby phosphorylation enables the inactivation of the NLRP3 inflammasome. Moreover, it is not yet defined whether this PKA-dependent inactivation mechanism is globally applicable to other NLRP family members or to other NLRs.

The consensus sequence of PKA is [R/K](2)-X-[S/T]-[Hydrophobic], due to two glutamic acid residues in the catalytic pocket of PKA that form ionic binding sites for the P-3 and P-2 position of the substrate, as well as a hydrophobic pocket that favours a hydrophobic residue in the P+1 position[56,57,58]. This sequence conforms to the catalytic site of PKA and confers specificity to the kinase. The sequence surrounding the Ser295 residue of NLRP3 is highly conserved among different species (Figure 3A), yet differs from the canonical PKA consensus sequence at the P+1 position, where a basic residue is found in all mammalian NLRP3 orthologues examined, and 12 of the 14 human NLRP family members (Figure 3B). Another interesting feature of the sequence surrounding Ser295 is the proline residue located at the P-1 position, which while conserved in orthologous mammalian NLRP3 proteins, is unusual for PKA phosphorylation sites. Nonetheless, the high level of conservation in this region across mammals suggests the importance of this site for the regulation of NLRP3 activity.

3.3 Protein kinase D can also phosphorylate NLRP3 at Ser295

A series of investigations revealed that PKD could effectively target both mouse and human NLRP3 at the Ser295 residue. The sequence surrounding the Ser295 site in human NLRP3 conforms to the prototypical PKD consensus sequence (i.e., [I/L]-X-[R]-X-[S/T]), where a hydrophobic Ile/Leu residue at the P-5 position and a basic Arg residue at P-3 define the optimal sequence for PKD-mediated phosphorylation[87]. While the Ser phosphorylation site and Ile/Leu residues are conserved among all but one of the mammalian NLRP3 orthologues examined, the Arg residue at P-3 is absent in all but mouse and hominid NLRP3 proteins (Figure 3A). Taken together, these observations suggest that the PKD-dependent mechanism for NLRP3 regulation is narrowly restricted to only a few organisms. Additional biochemical analyses will be required to determine if other NLRP3 homologues that lack the consensus sequence are effective targets of PKD. For example, it is possible that the conservation of a basic residue (Lys/Arg) at the P-2 position among the majority of NLRP3 mammalian homologues may accommodate the lack of Arg at P-3.

In contrast to the inhibitory effect reported in the studies of PKA-dependent regulation described in Section 3.2, the phosphorylation of the same Ser295 site by PKD was necessary for inflammasome activation (Figure 2B)[46]. In response to inflammasome activators including ATP, nigericin, as well particulates Alum and Nano-SiO2, mitochondria-associated ER membranes (MAMs) clustered around Golgi membranes, where diacylglycercol (DAG) production was enhanced, thereby activating PKD in MAMs. A Golgi integrity disruption agent, Brefeldin A, attenuated NLRP3 activation, indicating the importance of Golgi signaling in close proximity to MAMs for NLRP3 activation. NLRP3 agonists also resulted in NLRP3 translocation to MAMs, where the proximate PKD phosphorylated NLRP3 at Ser295

in the NACHT domain. Inhibition or knock-down of PKD specifically blocked NLPR3 inflammasome activation, and PKD activity was required for NLRP3 inflammasome activation *in vivo*. Phosphorylation of NLRP3 by PKD resulted in the release of NLRP3 from Golgi-associated MAMs to the cytosol, where it assembled a fully mature inflammasome. Blocking PKD signaling resulted in NLRP3 retention at MAMs and the abrogation of NLRP3 inflammasome signaling. Additionally, inhibiting PKD prevented NLRP3 activation in cells derived from patients with NLRP3 mutations resulting in CAPS.

It is apparent that in response to NLRP3 activators, MAMs cluster around the Golgi, NLRP3 is translocated to these membranes, where increased DAG triggers PKD to phosphorylate NLRP3. What was initially unclear was how a phosphorylation event at identical sites by different kinases could have such contradictory effects on NLRP3 activity, and could have been explained by either the subcellular localization of NLRP3, or by the temporal regulation of inflammatory signaling and NLRP3 activation. Since both particulate agonists like Alum and Nano-SiO2, considered to be "slow-acting" inflammasome agonists (>4 hours) and ATP and nigericin, considered to be "fast-acting" inflammasome agonists (<1 hour) both result in similar localization events, PKD and PKA signaling, it does not appear that the rate of NLRP3 agonist signaling plays a role in differentiating these phosphorylation events. Because PKD inhibition had no effect on PKD enrichment, but NLRP3 release from MAMs was abolished, it appears the phosphorylation activity of PKD toward NLRP3 was the crucial and necessary step in this process of cytosolic translocation, recruitment of ASC and downstream inflammasome assembly. It appears that the subcellular localization of NLRP3 and these kinases explicates these differences, and that PKD activity within the MAMs is sufficient and necessary for release of NLRP3 to the cytosol, but that cytosolic phosphorylation by PKA at that same site is inhibitory on NLRP3 catalytic activity and downstream nucleation of the inflammasome.

3.4 Phosphorylation of Ser295 as a global regulator of NLRP family members

The PKA- and PKD-dependent phosphorylation of Ser295 is evidently important for the regulation of human NLRP3 inflammasome activity; however, the phosphorylation site is not conserved among the other NLRP family members. Indeed, only one other NLRP actually possesses a hydroxylic residue that aligns with the Ser295 located in the NACHT domain of NLRP3 (Figure 3B). This Ser252 residue within NLRP14 is not defined by the [R/K](2)-X-[S/T] or [L/I]-X-[R]-X-[S/T] consensus sequences, so it is unlikely to be phosphorylated by PKA or PKD respectively. Taken together, a survey of the entire NLRP family would suggest that the PKA- and PKD-dependent regulation of inflammasome activity via the Ser295 residue is restricted to NLRP3 and does not extend to the other family members. Despite that, multiple PKA and PKD phosphorylation sites are predicted *in silico* to occur within (or

proximal to) the NACHT domain of other NLRP family members (Figure 3C), as well as in other regions of the proteins, such as the LRR or PYD. This observation implies that other NLRPs could be phosphorylated by PKA or PKD, so additional biochemical analyses will be required to identify if prospective consensus sites for phosphorylation in all human NLRP proteins represent a conserved mechanism of regulation amongst the family.

Intriguingly, 8 of the 14 NLRPs display acidic amino acid substitutions at the Ser295 site (Figure 3B), suggesting that the incorporation of negative charge in this region of the NACHT domain may have a functional role in the regulation of inflammasome activity. It is possible to infer that most NLRP proteins are, therefore, held in an inhibitory conformation with the glutamic and aspartic acid residues providing similar molecular properties to that of the phosphorylated serine. While it is unclear if phosphorylation blocks ATP binding, Mortimer *et al*. demonstrated that Ser295 phosphorylation attenuated ATP hydrolysis and inflammasome activity, so the introduction of negative charge near the Walker B motif clearly impacts upon the active site. At least two NLRP proteins that contain a negative residue at the Ser295 site were previously reported to bind and/or hydrolyze ATP (i.e., NLRP1[24] and NLRP12[23]), so the presence of a negative residue in place of the serine does not support the complete abrogation of catalytic function. These findings suggest that while phosphorylation of Ser295 appears to play an important role in the regulation of certain NLRPs, it is likely that distinct enzymatic properties and regulatory mechanisms will be identified for the different family members.

4. The Phosphorylation of Ser295 and Its Impact on NLRP3 Structure

4.1 Molecular Modeling of the NLRP3-NACHT Structure

To further examine the inhibitory effects of PKA phosphorylation of Ser295 on NLRP3 activity, a structural homology model of the NLRP3 NACHT domain was assembled with the Phyre2 Protein Fold Recognition Server[59]. Despite concerted efforts to crystalize different NLRP proteins or close family members, no 3-dimensional structure of any NLRP-NACHT domain has been published, and this knowledge gap represents a critical impediment to understanding the activation mechanisms of these inflammatory platforms. Despite this obstruction, significant advances in computational techniques have resulted in more accurate protein structure predictions[60], and we have applied *in silico* modelling approaches to predict the effects of Ser295 phosphorylation on the topology of the nucleotide-binding site. Phyre2 uses remote homology detection methods to build structural models by matching the sequence of interest to libraries of known folds, minimizing the more error-prone aspects of simulated-folding approaches[59]. In an NLRP3-NACHT structural model derived with Phyre2 (Figure 4), 98% of

NLRP3 NACHT residues were modeled at over a 90% 'confidence' level (i.e., represents the probability that the match between sequence and the constructed model is a true homology). Residues modeled at lower scores were the first seven residues and the last residue of the submitted sequence string for the NLRP3 NACHT domain (residues 190-197 and 536, respectively). This is likely due to the expansion of flexibility given these residues were removed from any neighboring structural constraints.

Three protein structures were selected based on heuristics to model the NLRP3-NACHT domain, including: NLRC4 (PDB 4KXF), also a caspase-1 activating inflammasome protein, NAIP5 (PDB 5YUD), a sensor component of the NLRC4 inflammasome, and a NAIP5-NLRC4 complex (PDB 6B5B). Ultimately, the NLRP3 NACHT model was predicted to contain the following secondary structure: 52% α-helices, 11% β-strands, 5% transmembrane helices, and the remaining 15% intrinsically disordered/unstructured. Given the close alignment of gene ontology between the template structures and NLRP3 sequence in the modeled regions, we place reasonable confidence in the accuracy of predicted model; however, empirical data will obviously be required to define the native structure.

To determine the reliability of the NLRP3-NACHT model with respect to the core nucleotide-binding site structure, we directly compared the NLRP3-NACHT model to the published x-ray crystal structure of apoptotic protease-activating factor (APAF)-1, the protein closest to NLRP3-NACHT in sequence similarity with high-quality 2-3 Å rmsd published structure and reliable biochemical data on nucleotide binding and hydrolysis (PDB: 1Z6T, 2.21Å)[61,62,63]. APAF-1 is also a nucleotide-binding, inflammasome forming receptor, and it shares conservation of key functional motifs with NLRP3 (Figure 4A-B). Several residues of APAF-1 known to coordinate the bound nucleotide are not present within the NLRP3-NACHT model. However, most key residues were situated in close proximity within the alignment, and the overall topology of the catalytic pocket, which in turn plays a key stabilization role in nucleotide-binding, appears to be reasonably conserved given the differences in sequence (Figure 4C-E).

In the empirical APAF-1 structure, the specific binding of the nucleotide is achieved with direct hydrogen bonding from Val127, Gly157, Gly159, Lys160, Ser161, Val162 and His438. Direct hydrogen bonds from the amide and carbonyl of Val127 coordinate the N1 and N6 atoms of the adenine base (respectively), while the amides of Gly157, Gly159, Lys160 and Val162, as well as the amino group of Lys160 and the τ-nitrogen of the His438 imidazole group provide hydrogen bonding with the β-phosphate. The final direct H-bond is between the amide of Val162 and the α-phosphate. Water-mediated H-bonds between the carbonyl groups of Gly159, Val125 and the side-chain of Arg129 stabilize the adenine base, while the carbonyl of Ser422 provides the final water-mediated H-bond with the ribose

moiety. Lastly, van der Waals interactions with both the adenine and ribose groups are provided by Val127, Gly159, Val162, Arg129, Pro321 and Leu322.

Most of the direct hydrogen bonding residues in APAF-1 do align with conserved or similar residues in NLRP3 (i.e., Tyr381, Gly229, Gly231, Lys232, Thr233, Ile234 and Arg351). Likewise, APAF-1 residues that may confer structural stability in the active site also align well with the NLRP3 model. These include the residues which provide water-mediated hydrogen bonds, Val125, Arg129 and Ser422 in APAF-1 (aligning with NLRP3 Tyr381, Lys377, and Thr430, respectively), and those which provide only van der Waals interactions: Pro321 and Leu322 in APAF-1 (aligning with NLRP3 Pro412 and Leu413, respectively). According to the widely-used amino acid substitution matrix BLOSUM62, two of these substitutions possess low scores: the Tyr substitutions for Val (i.e., APAF-1 Val127 and Val125 replaced with Tyr381 and Tyr385 in NLRP3), and Arg substitutions for His (i.e, APAF-1 His438 replaced with Arg351 in NRLP3). It is possible that the homology model of NLRP3 could still function to provide equivalent bonding patterns, since both interactions from Val127 and Val125 in APAF-1 are provided by the amino-acid backbone (carbonyl and main chain, respectively) of the NLRP3 model, and the hydrogen-bond between APAF-1 His438 and the β-phosphate of the nucleotide could be substituted by the NLRP3 Arg351 side-chain.

Properties inherent to the NACHT domain, namely ATP-binding and ATP-hydrolysis, are indispensable for NLRP3 oligomerization and inflammasome assembly. Since all of the aforementioned motifs (Section 1.3) play key functional roles in the regulation of substrate binding and catalysis, we postulated that PKA-dependent phosphorylation of Ser295 could result in conformational fluctuations within these structured regions. A phosphoryl group was introduced at the Ser295 residue within the NLRP3-NACHT model structure (Figure 5A, Ser295 in cyan; Figure 5B, pSer295 in green) using the Vienna-PTM Server[64,65]. Vienna-PTM utilizes several different widely-used and extensively-tested molecular dynamics (MD) simulation force fields, as well as integrates newly-derived parameters for phosphate ions within the GROMACS 4.5 force field parameter sets selected[66]. To minimize the energy and identify the most stable conformation of our phosphorylated model structure, we applied the obminimize program in openbabel[67,68]. The structure was minimized using the steepest descent algorithm. The phosphorylated NLRP3-NACHT model was aligned with the unphosphorylated structure, resulting in an overall RMSD of 0.913 over 345 of 347 residues (Figure 5C). As expected, pairwise RMSD analysis suggests the largest shifts in fold conformation and residue position immediately preceding Ser295, but the introduction of the post-translational modification also resulted in higher energy deviations between key residues in the Sensor 1 and 2 regions, even affecting secondary structure in those areas, as well as in other concentrated areas throughout the structure (Figure 5D-F). These

deviations are representative of the impact that Ser295 phosphorylation has on secondary structure composition within these areas and suggests a disordering of regions previously composed of β-strands. As noted above, these regions play key roles in the activity of the NACHT domain as a whole, and these results could explain why phosphorylation of Ser295 results in both the abrogation of NLRP3 ATPase activity as well as an impediment to any future activation.

Lastly, we examined the effect of phosphorylation on the nucleotide-binding site using the pSer295 NLRP3-NACHT model derived with Vienna-PTM. Subtle, yet potentially important, deviations were observed (Figure 6A-B), including a positional shift in the location of the Lys232 residue of the Walker A motif and the Pro412 residue (Figure 6C-D). This proline is conserved in most of the NLRPs (all except NLRP2 and NLRP7), and mutation of this residue disrupts downstream signaling in NOD2[69]. Due to its known interaction with the adenine moiety of ATP in NOD2, and the known hydrophobicity of this region being essential in facilitating the binding of ATP, this shift could be significant in explicating the effects of phosphorylation on the inhibition of NLRP3 activity.

It is difficult to predict how small changes within the catalytic pocket could impact upon overall inflammasome activity, or whether conformational changes instigated by Ser295 phosphorylation even need to be conveyed within the catalytic pocket to result in inactivation. A subtle adjustment in Lys232 or Pro412 positioning, as observed with modelling in Figure 6, could result in impaired nucleotide-binding, but it is equally possible that the subtle conformational shifts within the catalytic pocket have no functional impact, and the inhibitory effect of pSer295 actually occurs in other regions distal to the core motifs of the ATP-binding pocket. Experiments to individually interrogate the impacts of Ser295 phosphorylation on ATP-binding, ATP-hydrolysis and ADP-release properties of the NLRP3 inflammasome will be essential to understand the role of ATP in regulating NLRP3 activity. While ATP-binding appears indispensable for NLRP3 activation, it remains unclear if it is ATP-binding, the cleavage of the β-γ phosphate bond, the release of the phosphate or the release of the ADP moiety that drives the conformational changes necessary to expose docking regions for NLRP3 receptor oligomerization and/or other inflammasome-binding partners (such as ASC). In addition, empirical structural data to determine where each of these key residues are positioned will be important in understanding exactly how the phosphorylation of Ser295 impacts upon the conformation of the domain, therefore negatively regulating inflammasome activity.

5. Conclusions and Future Opportunities

While studies offer reasonable evidence to support a major role for PKA (and PKD) in NLRP3 regulation, several matters remain unexplored. Atomic resolution structural data are available in the PDB for several domains of the NLR family, including structures for the PYD of NLRPs 1,3,7,10,12 and 14, the LRR of NLRP1, the CARD of NOD1 and NLRP1, the BIR of NAIP, as well as the RNA-binding element of NLRX1. Unfortunately, no structures have been solved for the central catalytic NACHT domain of any NLRP protein. So, additional investigations of the NACHT domain structural architecture, the conformational status of the domain in relation to nucleotide-binding and/or phosphorylation state, and the residues involved in oligomerization will be essential for a complete understanding of NLRP inflammasome function.

It has been widely assumed that the enzymatic properties of the nucleotide-binding NACHT domain are fundamental to the functionality of all 14 NLRP members, inflammasome assembly and otherwise, but ATP-binding and/or hydrolysis has only been demonstrated with NLRP3[21], NLRP7[22] and NLRP12[23]. This lack of experimental data for NLRP3-NACHT ATP binding, hydrolysis and oligomerization has to date obstructed the ability to develop an overall understanding of the biochemical mechanisms of inflammasome activation. Multiple publications concerning inflammasome activation appear to have misconstrued results in the seminal 2007 publication by Duncan by reporting that hydrolysis was established as a critical step in inflammasome function, whereas the data only demonstrated that ATP binding was indispensable for downstream signaling and did not conclusively link ATP hydrolysis with inflammasome activation[21]. While investigators have stated that it is probable that ATP binding and/or hydrolysis in the NACHT domain is/are responsible for a conformational remodeling that is transferred to other NLRP domains, overall there is much to learn regarding the functionality of the NACHT activity its role in inflammasome oligomerization, activation, and downstream signaling.

NLRP3 protein can be maintained in an inactive yet signaling competent state by protein-protein interactions with the ubiquitin ligase–associated protein (SGT1) and heat-shock protein 90 (HSP90)[70] (Figure 7A). Knockdown of SGT1 by small interfering RNA or pharmacological inhibition of HSP90 with geldanamycin was demonstrated to abrogate inflammasome activity and reduce NLRP3-mediated disease symptoms in mice. Additionally, SGT1 and HSP90 form similar complexes with several other NLRs (NLRP2,4,12, NOD1,2 and IPAF), and are indispensable for NOD2 and IPAF inflammasome activation as well. Notably, HSP90 but not SGT1 was crucial for the maintenance of stable NLRP3 protein levels in the cell. Treatment with geldanamycin resulted in a substantial decrease in endogenous THP-1 NLRP3 levels, and also provided complete depletion of NLRP3 by 8h of treatment, and thus to

the inhibition of downstream inflammasome activity, in 'Flp-In T-rex' HEK293T cells with inducible NLRP3 expression. These effects were blocked by the proteasome inhibitor lactacystin, strongly suggesting that the loss of HSP90 function resulted in the proteasome-dependent degradation of NLRP3. Hydrolysis of ATP in NLRP3 could aid in driving a conformational change that exposes docking sites for nucleation of the inflammasome and regulatory partners, but which binding partners are essential, and the order in which they bind remains unknown. Furthermore, the integration of phosphorylation into these schemes remains to be considered.

In consideration of the data available for phosphorylation of Ser295, we propose a scheme in which NLRP3 is serially phosphorylated and dephosphorylated to regulate inflammasome signaling in response to priming (Figure 7A), activating stimuli (Figure 7B), and assembly (Figure 7C). This mechanism of regulation is not unique to NLRP3; the pyrin inflammasome is also regulated by phosphorylation at Ser242 and bound by inhibitory 14-3-3 proteins, which must be relieved by phosphatase activity prior to inflammasome assembly[71,72,73,74]. PKA stimulation and the ensuing phosphorylation of Ser295 is inhibitory prior to activation, and can also initiate inflammasome disassembly of the mature inflammasome. PKD phosphorylation of that same residue is essential for the progression of NLRP3 through the activation mechanism, and drives the subcellular relocation of NLRP3 from Golgi-associated MAMs to the cytosol where the inflammasome complex is assembled. The intrinsic phosphorylation status of Ser295 in the priming stage remains unclear, but PKA was found to be associated with NLRP3 at that stage[49]. Additionally, NLRP3 is ubiquitinated with mixed K48 and K68 chains at this phase, and Ser295 phosphorylation is known to play a key role in ubiquitination[50]. Therefore, we propose that NLRP3 is likely phosphorylated at Ser295 in the priming stage, driving ubiquitination and blocking oligomerization at resting state. Following the sensing of a signal two agonist, it is unclear whether NLRP3 would be dephosphorylated by some unknown phosphatase prior to migrating to Golgi-associated MAMs where PKD activity is enhanced, or if PKD and PKA activities on this site are redundant. PKD phosphorylation of NLRP3 is plainly indispensable for NLRP3 release from these membranes to the cytosol, although dephosphorylation of Ser295 is obligatory for NLRP3 inflammasome assembly. The co-localization of PKA and NLRP3 was not considered in any study of Ser295 phosphorylation. The seemingly specific inhibition of PKD in MAMs did abolish NLRP3 signaling; and therefore, each kinase may regulate NLRP3 in distinctive cellular compartments along the activation pathway. Following activation, the experimental stimulation of PKA and NLRP3 phosphorylation could induce dissociation of the inflammasome, but whether this mechanism of action is biologically relevant remains uncertain.

Because the bulk of investigations into NLRP3 phosphorylation mechanisms were performed *in vitro*, determining what mechanisms are germane to intrinsic NLRP3 activation *in vivo* will require further exploration in the relevant model systems. Furthering our understanding of the biochemical and biophysical properties of NLRP3 and other inflammasome forming proteins will be fundamental to the development of treatments for the myriad of human diseases in which these proteins play central roles, including CAPS, multiple sclerosis, lupus, the crystalline deposit-related diseases of silicosis and gout, inflammatory bowel disease, infectious colitis, metabolic diseases (including obesity, type II diabetes, and atherosclerosis), Parkinson's disease, and renal disease (acute kidney injury and chronic kidney disease)[75,76,77]. Multiple NLRP3 inhibitory compounds are under investigation[78,79,80], and it is hoped that these research endeavors will ultimately guide the attainment of treatment for many chronic inflammatory pathologies.

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7. Author Contributions

CFS completed the molecular modelling of NLRP3 NACHT and Ser295 phosphorylation, wrote the manuscript, and prepared the figures. JAM edited the manuscript, supervised trainees and provided intellectual contributions to the project. Both authors reviewed the results and approved the final version of the manuscript.

8. Authors' Competing Interests Statement

JAM is cofounder and holds an equity position in Arch Biopartners Inc. All other authors declare no conflicts of interest.

Figure and Table Legends

Table Legends

Table 1. Phosphorylation of the NLR Family Members. The nucleotide-binding domain and leucine-rich repeat-containing (NLR) family members are subdivided based on the presence of one of four N-terminal effector domains: NLRAs with an acidic domain, NLRBs with a Baculovirus IAP repeat, NLRCs with a caspase recruitment domain, or NLRPs with a pyrin domain. Empirical NLR phosphorylation events are listed and, if known, linked with the protein kinase associated with each site in the last column. The naming system approved by the HUGO Gene Nomenclature Committee (HGNC)[10] is used. Domain abbreviations: AD, acidic domain; BIR, Baculovirus inhibitor of apoptosis repeat; CARD, caspase activation and recruitment domain; CT, C-terminal; FIIND, function to find domain; LRR, leucine rich repeat; NACHT, nucleotide triphosphatase (NTPase) domain [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (in-compatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)]; NT, N-terminal; PYD, pyrin domain. Empirical phosphorylation and kinase data obtained from iPTMnet and PhosphoSite Plus[81,82].

Table 2. NLRP3 Phosphorylation Sites. The impact of several phosphorylation events on NLRP3 inflammasome assembly and downstream maturation of caspase-1 and IL-1β have been defined. Empirical NLRP3 phosphorylation sites and the corresponding human (UniProtKB: Q96P20) or mouse (UniProtKB: Q8R4B8) residues are listed, as is the domain in which the site is found. Dashed lines in the domain column represent disordered, linker regions. Results of the phosphorylation event, as well as the kinase or phosphatase associated with the event are listed with the corresponding reference in the low or high-throughput experiment column (LTP/HTP, respectively). Abbreviations used: LTP, low throughput methods (i.e., sites were identified and then validated with other biochemical methods), HTP, high throughput methods (i.e., sites were identified using only discovery mass spectrometry), PYD, pyrin domain; NACHT, [NAIP (neuronal apoptosis inhibitory protein), CIITA (MHC class II transcription activator), HET-E (in-compatibility locus protein from *Podospora anserina*) and TP1 (telomerase-associated protein)] domain; LRR, leucine-rich repeat domain; PKA (PRKACA), cAMP-dependent protein kinase; PKD (PRKD), serine/threonine protein kinase D; PTPN22, tyrosine-protein phosphatase non-receptor type 22; JNK1 (MAPK8), c-Jun N-terminal kinase 1; PP2A (PPP2CA), protein phosphatase type-2A catalytic subunit.

Figure Legends

Figure 1. **The Canonical Two-Signal Model for NLRP3 Inflammasome Activation**. The first transcriptional priming step (i.e., Signal One) in the assembly of the NLRP3 inflammasome is initiated when microbial molecules (e.g., LPS) or endogenous cytokines (not shown) lead to the upregulation of NLRP3 and pro-IL-1β transcription through Toll-like receptor (TLR)-dependent activation of the NF-κB signaling pathway. Other elements of the NLRP3 inflammasome, including ASC, caspase-1 and pro-IL-18 are constitutively expressed. NF-κB signaling also results in the activation of BRCC3, a metalloprotease that deubiquitinates NLRP3 prior to activation. In the second, oligomerization and assembly step, several Signal Two stimuli, including but not limited to ATP, ionophores like nigericin, and particulate matter can result in activation of NLRP3 and inflammasome assembly. Most of these stimuli induce K^+ efflux, and/or calcium flux which is essential and sufficient for NLRP3 activation, but mitochondrial dysfunction, including reactive oxygen species (ROS), oxidized mitochondrial DNA or externalization of cardiolipin (mitochondrial dysfunction) can also act as the second signal. Upon detection of the second signal in the cytosol, the NLRP3 inflammasome is activated in an ATP-dependent manner with oligomerization of multiple NLRP3 molecules and recruitment of ASC and pro-caspase-1. Proximity-induced proteolytic cleavage and activation of caspase-1 enables the subsequent proteolysis and maturation of pro-inflammatory cytokines (i.e., pro-IL-1β and pro-IL-18) to their secreted forms, which can induce acute inflammatory responses in the surrounding tissue.

Figure 2: Mechanisms for PKA- and PKD-dependent regulation of NLRP3 inflammasome activity. **(A) PKA dependent regulation:** Prostaglandin E2 (PGE2) signaling (1) via the PGE2 receptors EP4 and EP2 activates adenylyl cyclase (ADCY), which catalyzes the conversion of ATP to 3',5'-cyclic AMP (cAMP)[51] and activation of PKA; resulting in NLRP3 phosphorylation. Comparably, bile acid signaling (2) via the transmembrane G-coupled receptor-5 (TGR5), and (3) dopamine binding to its dopamine D1 receptor (DRD1) stimulate cAMP production and induce NLRP3 phosphorylation[52,54]. The G protein-coupled calcium-sensing receptor (CaSR) mediates signal transduction (4) in response to extracellular DAMPs such as Ca^{2+} ions and ATP[55]. Activation of the receptor triggers the phospholipase C (PLC) catalyzed cleavage of phospholipid membrane component phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG – see panel B). IP_3 interaction with its receptor (IP_3R) on the endoplasmic reticulum induces Ca^{2+} ion release, and an increase in cytoplasmic Ca^{2+} concentration. In addition, CaSR activation inhibits ADCY, reducing cAMP levels. Both an increase in Ca^{2+} concentration and/or a decrease in cAMP levels results in NLRP3 inflammasome activation.

Additionally, two of these studies suggested that cAMP can directly bind NLRP3, triggering in the K48-chain ubiquitination of the LRR and NACHT domains by the Membrane Associated Ring-CH-type finger 7 (MARCH7) E3 ubiquitin ligase, aggregation and finally autophagy mediated NLRP3 degradation (3,4). **(B) PKD dependent regulation:** In response to inflammasome activators, including ATP, nigericin, and particulates Alum and Nano-SiO_2 (5), mitochondria associated ER membranes (MAMs) localize adjacent to Golgi membranes, where diacylglycercol (DAG) production was enhanced, thereby activating PKD in MAMs. Brefeldin A (BFA), a Golgi integrity disruption agent, attenuated NLRP3 activation and signaling, indicating the importance of Golgi signaling close to MAMs in NLRP3 activation. These agonists also resulted in NLRP3 translocation to MAMs, where the proximate PKD phosphorylated NLRP3 at Ser295 in the NACHT domain. Phosphorylation of NLRP3 by PKD resulted in the release of NLRP3 from Golgi-associated MAMs to the cytosol, where it assembled into a mature inflammasome. NLRP3 is predicted to be dephosphorylated upon activation, by a yet unknown phosphatase. Ultimately, caspase-1 is activated by the mature inflammasome and catalyzes the cleavage and processing of pro-IL-1β and IL-18. These mature cytokines are, in turn, secreted from the cell to stimulate inflammatory responses in the surrounding tissue.

Figure 3. Conservation of the Ser295 phosphorylation site and predicted PKA-dependent phosphorylation of NLRP family members. (A) Conservation of Ser295 among NLRP3 orthologues. Orthologous genes are listed in descending order by shared sequence identity. The conventional PKA consensus sequence and Walker B motif are labeled above the alignment, with the phosphorylated residue indicated (Ser295 in human NLRP3). Sequence conservation among species is denoted by the coloured highlighting when conservation of the amino acid class was retained among 4 or more species. Hydrophobic residues (A,I,L,M,F,W,V,C,G,P) are coloured in yellow; polar residues in green; hydroxylic (S,T,Y) in dark green and other polar (N,Q) in light green; acidic (D,E) in red; and basic (K,R,H) in blue. (**B)** Conservation of Ser295 among different NLRP family members. The PKA consensus sequence and Walker B motif are labeled above the alignment. The sequence logo below the alignment indicates sequence conservation among species, from increased conservation of amino acids and function (larger letters) to when amino acids were different and no conservation was retained (smaller letters). (**C)** Occurrence of PKA- and PKD-dependent phosphorylation sites based on Group-based Prediction System (GPS; http://gps.biocuckoo.org) analysis of the primary sequences of NLRP family members. The GPS 3.0 Species Specific Tool (Homo sapiens) was used to predict kinase-specific phosphorylation sites in hierarchy with medium threshold (6% false-positive rate)[88].

Figure 4. Structural modeling of the NACHT domain of NLRP3. A homology model of an extended NLRP3-NACHT domain (I190-L536) was generated *in silico* using Phyre2 and aligned with the X-ray crystal structure of APAF-1 (PDB: 1Z6T)[59,61]. The Phyre2 algorithm indicates that 98% of residues were modelled at >90% confidence (i.e., residues 190-196 and 536 were modeled *ab initio*, which can be unreliable, while the rest were modeled with high confidence, percentage indentity and alignment coverage). Both the NLRP3-NACHT model and the empirical APAF-1 structure were visualized using Pymol v2.2.1. The APAF-1 structure and the NLRP3-NACHT model display conserved topology within the nucleotide-binding domain. In (**A**, ribbon diagram) and (**B,** cartoon representation), APAF-1 is represented in gray, while the newly generated NLRP3 model is shown in purple. An ADP molecule empirically identified within the NACHT fold of APAF-1 is shown in red. Experimentally defined nucleotide-binding residues in APAF-1 are shown in orange, and the corresponding NLRP3 residues expected to interact with the nucleotide are shown in yellow. In (**C**), an alignment of key nucleotide-binding residue side chains between APAF-1 and the NLRP3-NACHT model is indicative of conservation within the nucleotide-binding domain of APAF-1 (**D**) and NLRP3 (**E**).

Figure 5. Predicted effect of Ser295 phosphorylation on the NLRP3-NACHT structure. Conserved sequence motifs that are important for nucleotide-binding and inflammasome activation are highlighted in cartoon diagrams for unphosphorylated (**A**, model in blue, Ser295 residue in cyan) and phosphorylated (**B**, model in dark red, pSer295 residue in dark green) NLRP3-NACHT domains. These include the Walker A (WA), Walker B (WB), Sensor 1 (S1) and Sensor 2 (S2) motifs, coloured as listed in the table at the bottom right hand corner of panel F). These regions play important roles in stabilizing intramolecular interactions, coordinating nucleotide-binding, nucleotide-hydrolysis, and protein conformational changes during oligomerization. In (**C**), an alignment of NLRP3-NACHT and pSer295-NLRP3-NACHT is coloured by RMSD. The overall RMSD of both structures is 0.913. Dark blue indicates close structural alignment, while higher energy deviations are shown in red. In (**D**), the NLRP3-NACHT sequence is coloured by RMSD as shown in panel C, indicating the predicted structural differences between the models. Key motifs are indicated above the sequence, and nucleotide-binding residues are labeled with black dots. In (**E**), an alignment of NLRP3-NACHT and pSer295-NLRP3-NACHT model structures illustrates conformational and positional changes of key motifs (**F**) upon phosphorylation of Ser295.

Figure 6. Potential impact of Ser295 phosphorylation on residues that coordinate nucleotide-binding. Representative structural models of NLRP3-NACHT (A) and pSer-NLRP3-NACHT (B) are

provided with active site residues shown in yellow and green for the unphosphorylated (coloured in blue) and phosphorylated NLRP3 (coloured in dark red), respectively. An ADP molecule (in red) was docked within the active site of each. The Ser295 and phosphorylated pSer295 residues are highlighted in cyan and dark green, respectively. Phosphorylation of the NLRP3 model *in silico* was performed with Vienna PTM, and the resulting structure was minimized with openbabel, using the steepest descent algorithm of obminimize. An alignment of NLRP3-NACHT and pSer295-NLRP3-NACHT structures (**C**) suggests gross conformational alterations of the ATP-binding pocket upon phosphorylation. Small positional and conformational changes are apparent for key residue side chains implicated in nucleotide-binding (**D**), which could contribute to the known disparities in function between unphosphorylated and phosphorylated NLRP3.

Figure 7. Involvement of Ser295 phosphorylation in the activation of NLRP3 inflammasomes. **(A) NLRP3 Priming (Signal One)**. Inflammasome components ASC, caspase-1 and IL-18 are constitutively expressed, while basal NLRP3 and pro-IL-1β transcription are inadequate for inflammasome activation[83]. Priming or transcriptional activation is facilitated by NF-κB signaling, including but not limited to NOD1/NOD2 activation of NF-κB, TLR signaling, and activation of the cytokine receptors TNFR1 and/or IL-1R1. Following transcription, the resting NLRP3 protein localizes adjacent to endoplasmic reticulum (ER) structures[84,85]. At this stage, NLRP3 may be held in an inactive but signaling competent complex with HSP90 and SGT1[70]. NLRP3 association with HSP90 blocks the proteasomal degradation of NLRP3. Inactive NLRP3 carries mixed K48 and K63 ubiquitin chains primarily on the LRR and NACHT domains. NF-κB signaling activates BRCC3 deubiquitination activity during priming, a requisite for NLRP3 activation[86,44]. Ca^{2+} signaling can inhibit NLRP3 activation through the CaSR/ADCY/cAMP axis since direct binding of cAMP to NLRP3 blocks activation[52]. Stimulation of the EP2/EP4 or TGR5 receptors activates protein kinase A (PKA) and induces phosphorylation of Ser295 in the NACHT domain of NLRP3, driving ubiquitination and inflammasome inhibition. PKA can associate with NLRP3 at this juncture as well, although the basal phosphorylation state of NLRP3 is still unresolved.

(B) NLRP3 Activation (Signal Two). NLRP3 inflammasome assembly is likely triggered by cellular stresses that converge upon K^+ efflux and Ca^{2+} flux rather than by direct PAMP or DAMP binding[37,87]. Pore-forming toxins, ionophores like nigericin, and the P2X7 receptor facilitate K^+ efflux and NLRP3 activation. Cell swelling can initiate mitochondrial dysfunction and Ca^{2+} flux through the transient receptor potential vanilloid 2 (TRPV2) receptor and/or voltage-dependent anion channel 1 (VDAC1) [88,89]. This culminates in release of intracellular NLRP3 agonists, including mitochondrial reactive oxygen

species (ROS), mitochondrial DNA (mtDNA) and cardiolipin. Crystalline DAMPs also impact on mitochondrial dysfunction, K^+ Efflux and Ca^{2+} flux via release of lysosomal cathepsins B and L. Both K^+ efflux and cathepsin B release are linked to dissociation of the HSP90-SGT1 complex from NLRP3[70]. P2Y2 receptor activation of PLC/IP_3/IP_3R signaling can trigger Ca^{2+} release from the ER, and coincident diacylglycerol (DAG) acts at mitochondria associated ER membranes (MAMs), where it provides downstream activation of protein kinase D (PKD). NLRP3 agonists also result in NLRP3 translocation to MAMs, and the proximate PKD phosphorylates NLRP3 at Ser295 in the NACHT domain.

(C) NLRP3 Inflammasome Assembly. Phosphorylation of NLRP3 by PKD results in the release of NLRP3 from Golgi-associated MAMs to the cytosol. NLRP3 is dephosphorylated upon activation by a yet unknown phosphatase. Active NLRP3 nucleates ASC, caspase-1 and other NLRP3 adapter proteins (not shown), before forming the mature inflammasome complex. Ultimately, caspase-1 activation catalyzes the processing and maturation of pro-IL-1β and IL-18. Protein kinase A (PKA) signaling downstream of EP2/EP4 or TGR5 receptors induces NLRP3 phosphorylation of Ser295 in the NACHT domain. The Ser295 phosphorylation may inhibit inflammasome activation or lead to inflammasome disassembly, depending upon the timing of the PKA stimulus. Inflammasome activation is presumed to require enhanced phosphatase (?) activity to ensure dephosphorylation of the inhibitory Ser295 site.

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[50] Phosphosite CST Curation Set 8345; Possemato A (2009); Biosample/Treatment: cell line, Jurkat/calyculin_A & pervanadate; Disease: T cell leukemia; SILAC: -; Specificities of Antibodies Used to Purify Peptides prior to LCMS: p[ST]P

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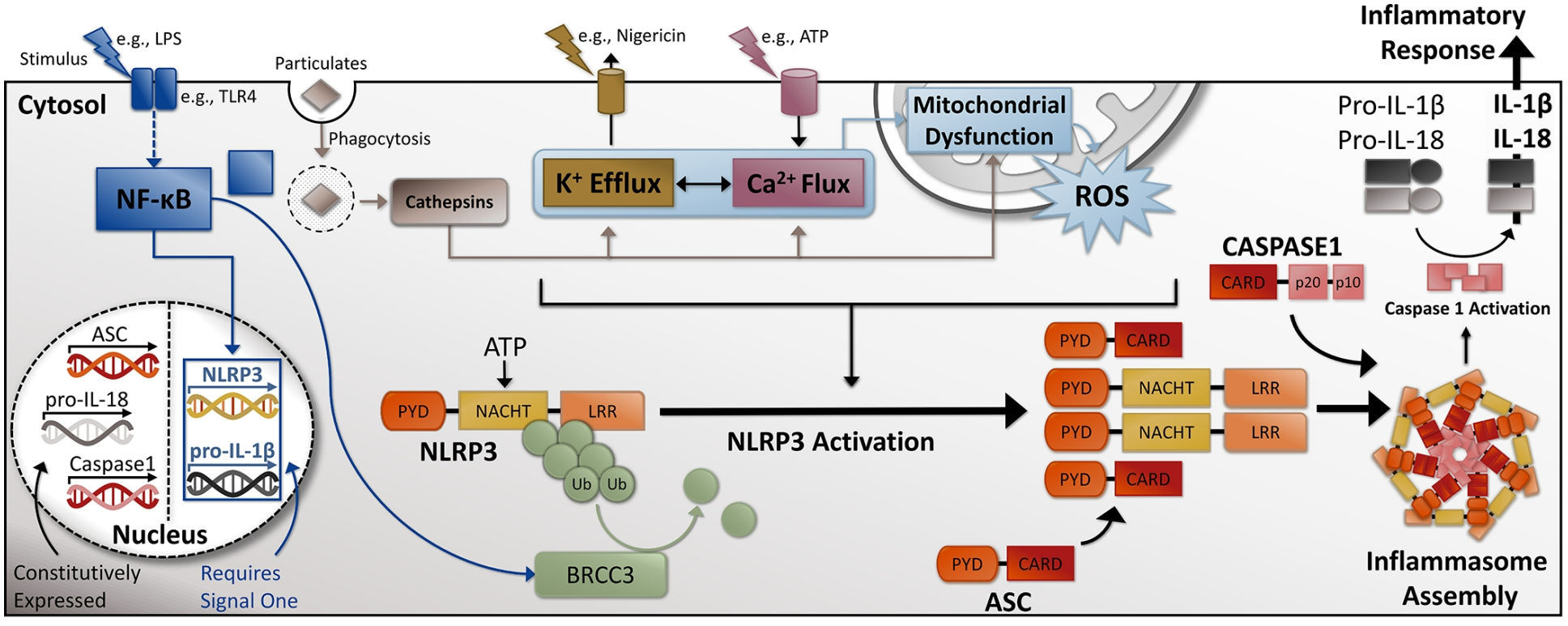
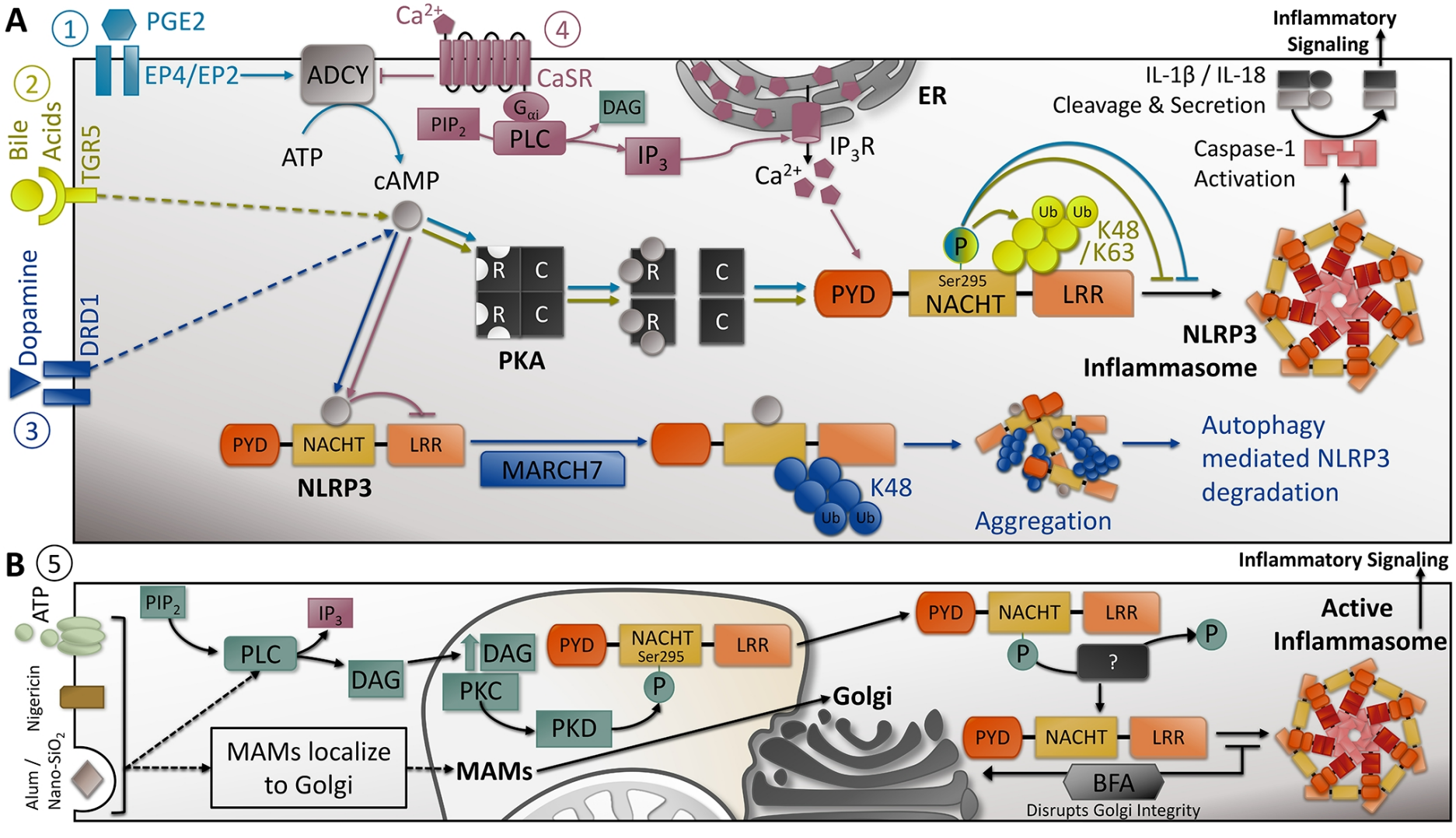


Signal One: Priming

Signal Two: NLRP3 Activation and Inflammasome Assembly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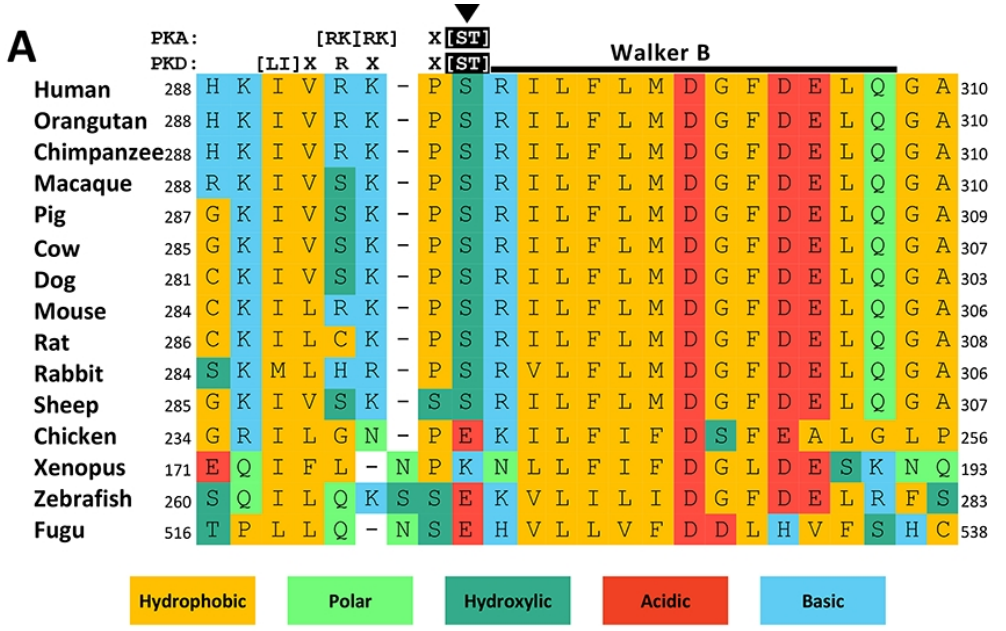
PGE2
EP4/EP2
ADCY
ATP
cAMP
2
Bile Acids
TGR5
3
Dopamine
DRD1
4
Ca2+
CaSR
Gαi
PIP2
PLC
DAG
IP3
ER
IP3R
Ca2+
R
C
PKA
PYD
NACHT
LRR
Ser295
P
Ub
K48
/K63
NLRP3
Inflammasome
Inflammatory Signaling
IL-1β / IL-18
Cleavage & Secretion
Caspase-1
Activation
MARCH7
K48
Aggregation
Autophagy
mediated NLRP3
degradation
B
5
ATP
Nigericin
Alum / Nano-SiO2
PIP2
PLC
IP3
DAG
PKC
PKD
MAMs localize
to Golgi
MAMs
Golgi
?
BFA
Disrupts Golgi Integrity
Active
Inflammasome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PKD: [LI]X R X X[ST]
Walker B
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Orangutan 288 HKIVRK-PSRILFLMDGFDELQGA 310
Chimpanzee 288 HKIVRK-PSRILFLMDGFDELQGA 310
Macaque 288 RKIVSK-PSRILFLMDGFDELQGA 310
Pig 287 GKIVSK-PSRILFLMDGFDELQGA 309
Cow 285 GKIVSK-PSRILFLMDGFDELQGA 307
Dog 281 CKIVSK-PSRILFLMDGFDELQGA 303
Mouse 284 CKILRK-PSRILFLMDGFDELQGA 306
Rat 286 CKILCK-PSRILFLMDGFDELQGA 308
Rabbit 284 SKMLHR-PSRVLFLMDGFDELQGA 306
Sheep 285 GKIVSK-SSRILFLMDGFDELQGA 307
Chicken 234 GRILGN-PEKILFIFDSFEALGLP 256
Xenopus 171 EQIFL-NPKNLLFIFDGLDESKNQ 193
Zebrafish 260 SQILQKSSEKVLILIDGFDELRFS 283
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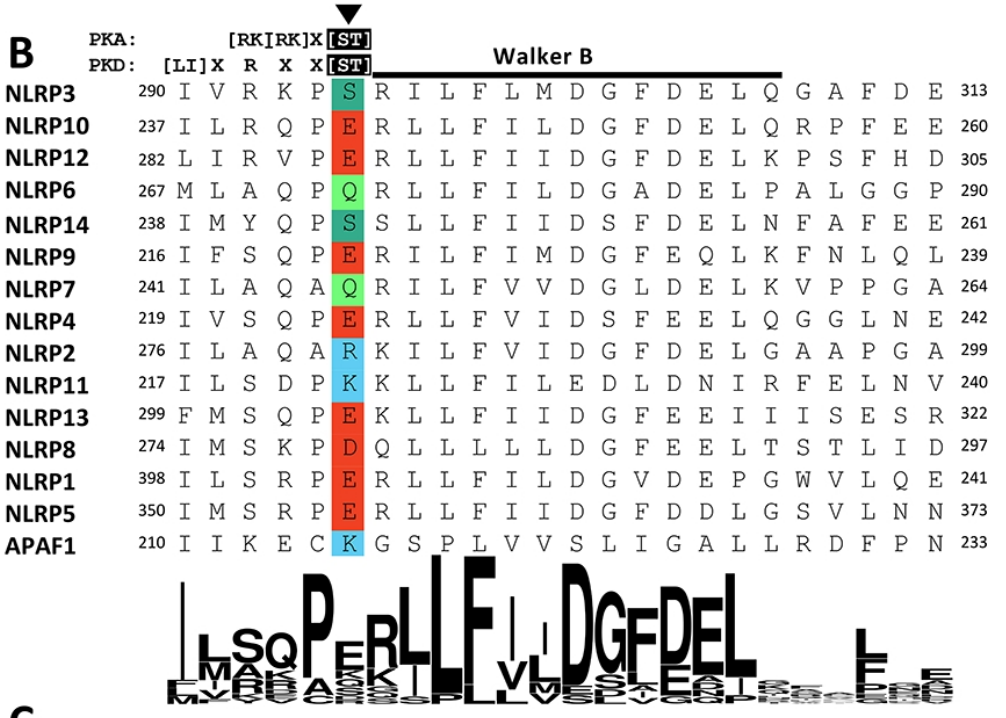
Polar
Hydroxylic
Acidic
Basic


B
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PKD: [LI]X R X X[ST]
Walker B
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NLRP12 282 LIRVPERLLFIIDGFDELKPSF HD 305
NLRP6 267 MLAQPQRLLFILDGADELPALG GP 290
NLRP14 238 IMYQPSSLLFIIDSFDELNFAF EE 261
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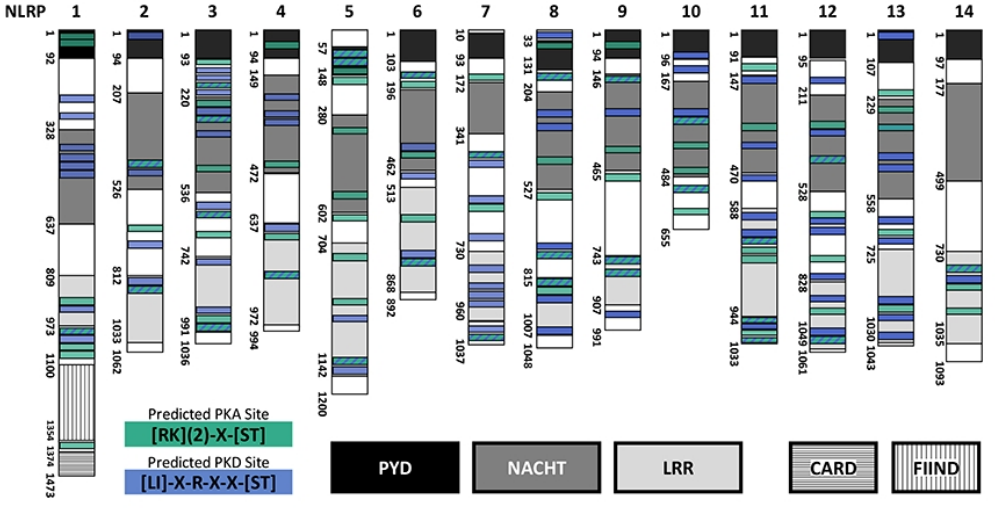
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Predicted PKD Site
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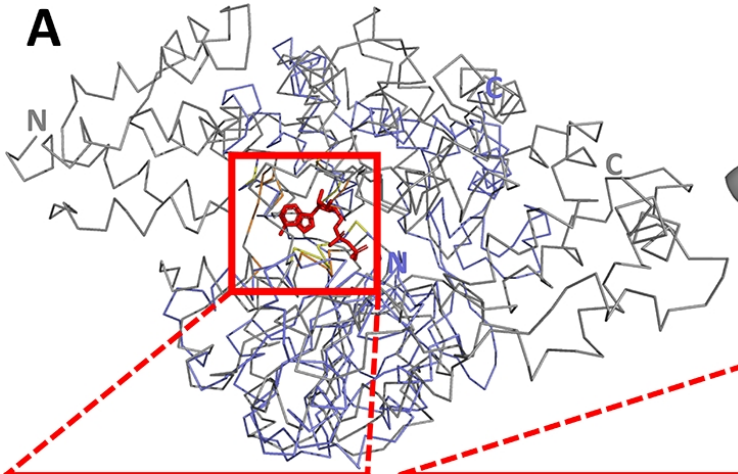
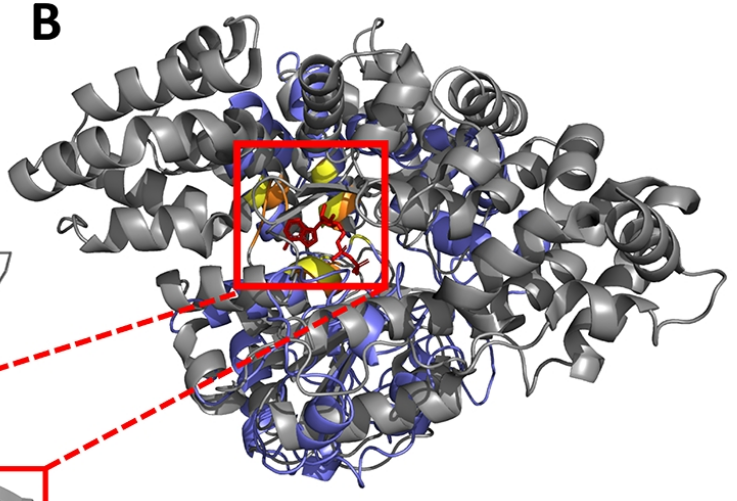
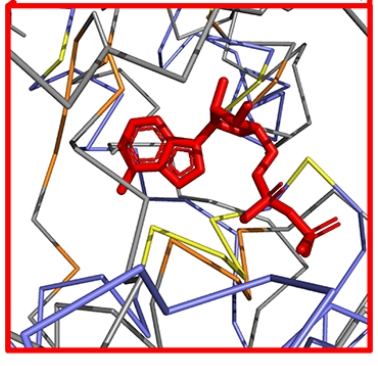
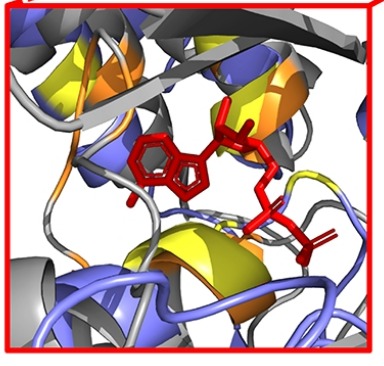
LRR
CARD
FIIND





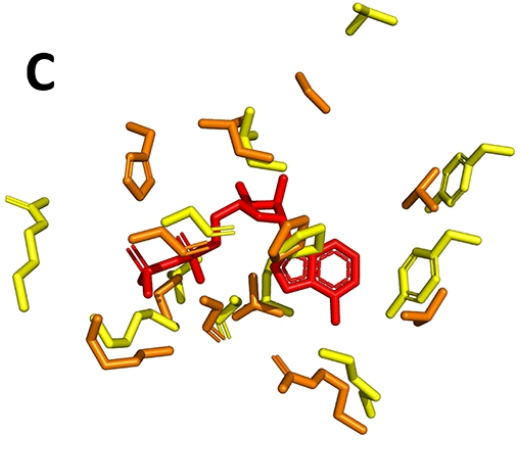


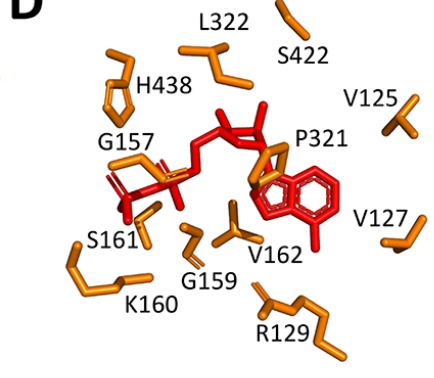


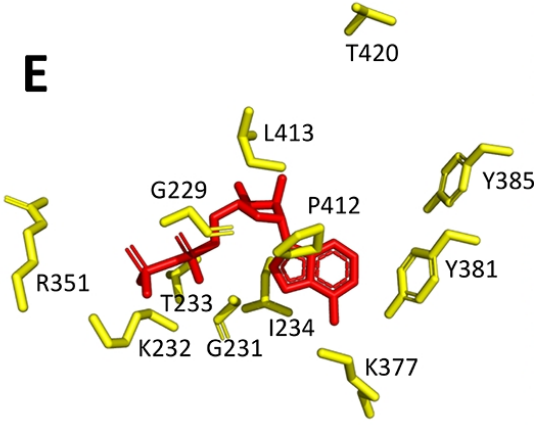
Nucleotide Binding Residues

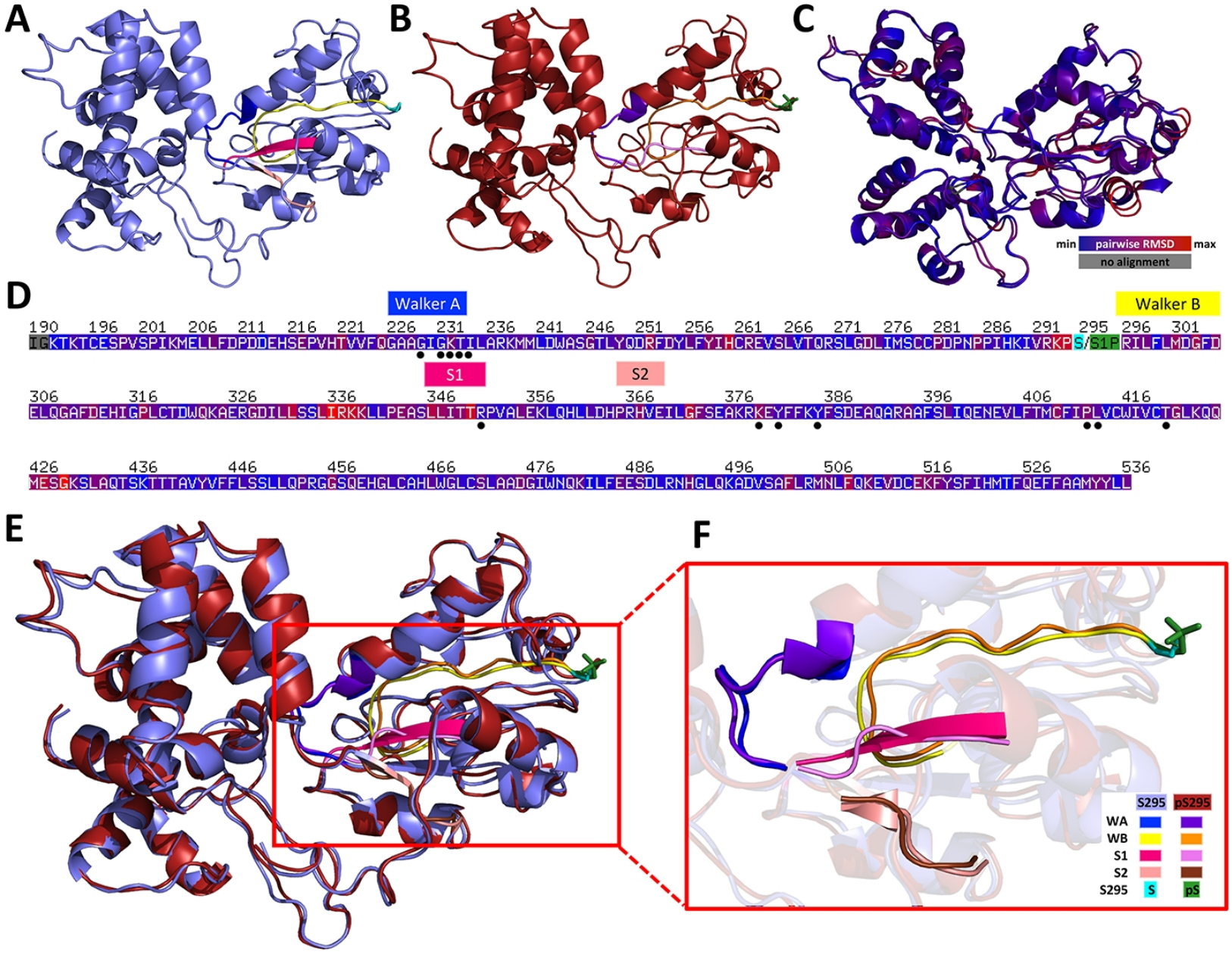
	H-Bonds						
APAF1	V127	G157	G159	K160	S161	V162	H438
NLRP3	Y381	G229	G231	K232	T233	I234	R351

	H_20 Mediated H-Bonds			VDW	
APAF1	V125	R129	S422	P321	L322
NLRP3	Y385	K377	T420	P412	L413





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B
C
min
pairwise RMSD
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no alignment
D
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Walker B
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306 316 326 336 346 356 366 376 386 396 406 416
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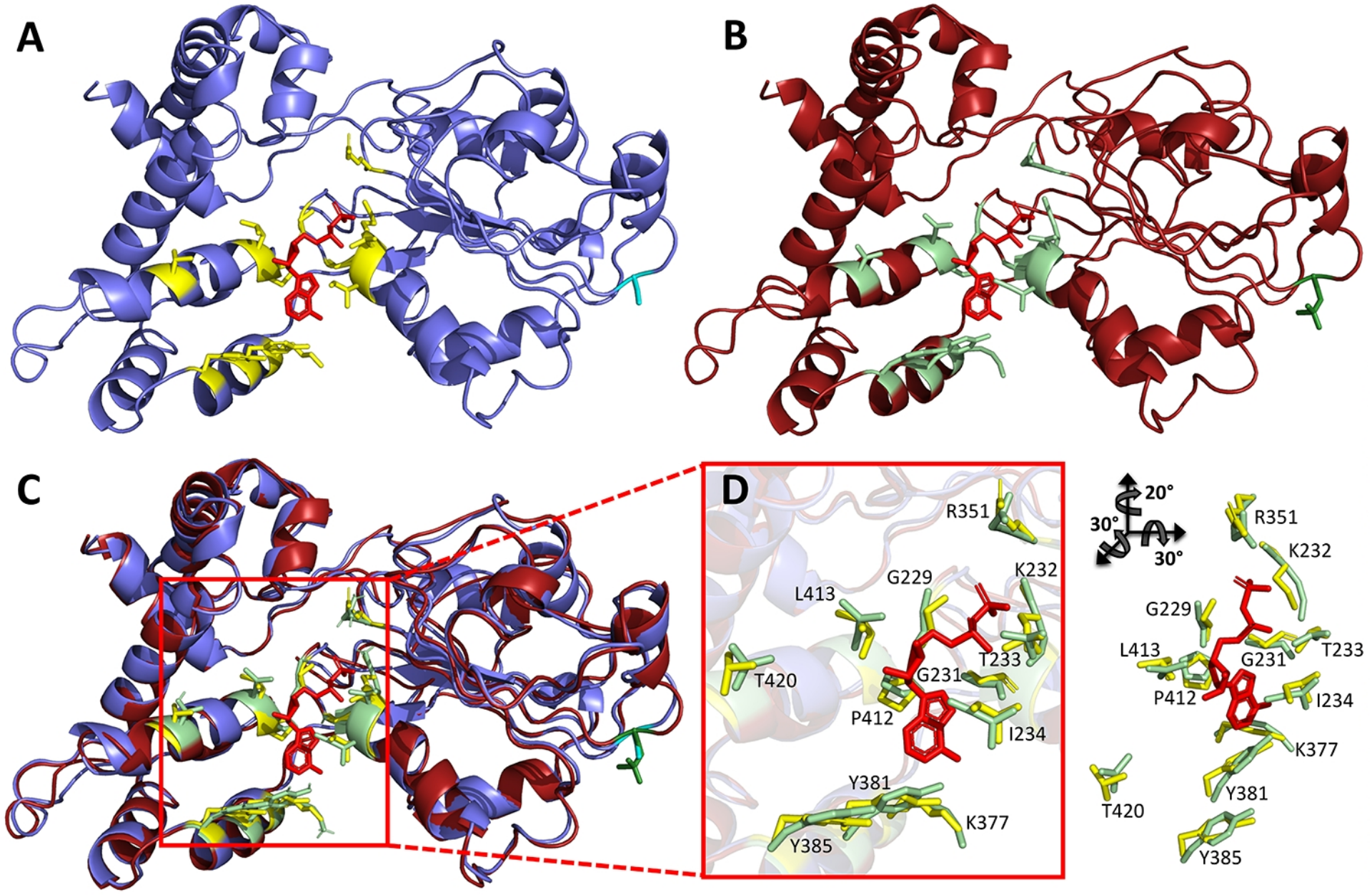
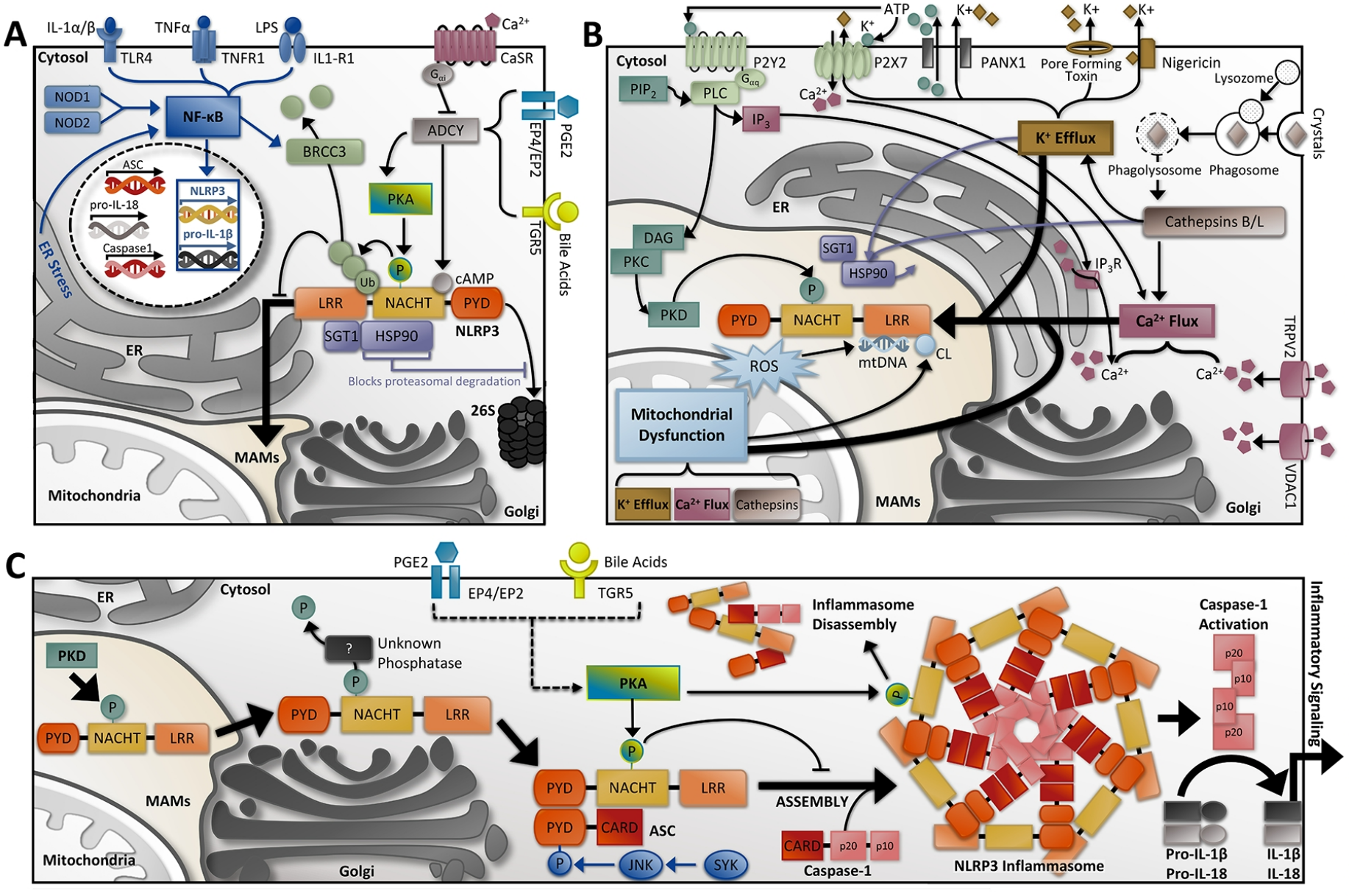
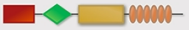
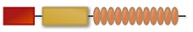
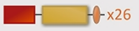
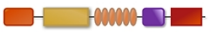
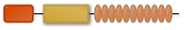
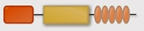
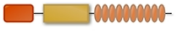
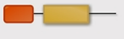
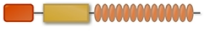
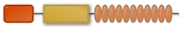
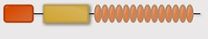
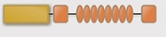
F
S295
pS295
WA
WB
S1
S2
S295
S
pS


A
B
C
D
R351
K232
L413
G229
T233
T420
G231
P412
I234
Y381
K377
Y385
20°
30°
30°
R351
K232
G229
L413
G231
T233
P412
I234
K377
Y381
T420
Y385


A
IL-1α/β
TNFα
LPS
Cytosol
TLR4
TNFR1
IL1-R1
Ca2+
CaSR
Gαi
NOD1
NOD2
NF-κB
BRCC3
ADCY
EP4/EP2
PGE2
TGR5
Bile Acids
ASC
NLRP3
pro-IL-18
pro-IL-1β
Caspase1
PKA
ER Stress
Ub
P
cAMP
LRR
NACHT
PYD
NLRP3
SGT1
HSP90
Blocks proteasomal degradation
ER
26S
MAMs
Mitochondria
Golgi
B
Cytosol
ATP
K+
P2Y2
P2X7
PANX1
Pore Forming Toxin
Nigericin
Lysozome
Crystals
PIP2
PLC
Gαq
Ca2+
IP3
K+ Efflux
Phagolysosome
Phagosome
ER
Cathepsins B/L
DAG
PKC
SGT1
HSP90
IP3R
PKD
PYD
NACHT
LRR
Ca2+ Flux
TRPV2
ROS
mtDNA
CL
Ca2+
Mitochondrial Dysfunction
K+ Efflux
Ca2+ Flux
Cathepsins
MAMs
Golgi
VDAC1
C
PGE2
EP4/EP2
Bile Acids
TGR5
ER
Cytosol
PKD
P
Unknown Phosphatase
?
PYD
NACHT
LRR
PKA
Inflammasome Disassembly
Caspase-1 Activation
MAMs
Mitochondria
Golgi
PYD
CARD
ASC
JNK
SYK
ASSEMBLY
CARD
p20
p10
Caspase-1
NLRP3 Inflammasome
Pro-IL-1β
Pro-IL-18
IL-1β
IL-18
Inflammatory Signaling

NLR Family	Protein		Domain Organization	Phospho - sites	Defined Phosphorylation Sites	Defined Kinases
NLRA	CIITA	class II, major histocompatibility complex, transactivator		14	S280[*†], S286, S288[*†], S293, S373[‡], S427, Y428, S434, T546, S834[#], T838, T947, S1050[#], S1073	MAPK1[*], MAPK3[†], GSK3B[‡], PRKACA[#]
NLRB	NAIP	NLR family, apoptosis inhibitory protein		10	S420, Y628, S932, S982, T987, Y989, S1043, S1051, T1056, S1398	
NLRC	NOD1	nucleotide-binding oligomerization domain containing 1		5	Y129, S217, T334, T797, S798	
	NOD2	nucleotide-binding oligomerization domain containing 2		14	T189, S238, T239, S402, T424, S425, S477, S493, S745, Y747, S983, S984, T1022, S1024	
	NLRC3	NLR family, CARD domain containing 3		6	S20, T49, S72, Y416, T751, S753	
	NLRC4	NLR family, CARD domain containing 4		2	S533[*], T736	PRKCD[*]
	NLRC5	NLR family, CARD domain containing 5		9	T185, S187, T727, S732, S923, S976, T1359, S1558, S1564	
NLRP	NLRP1	NLR family, pyrin domain containing 1		4	T41, S291, S823, T1000	
	NLRP2	NLR family, pyrin domain containing 2		8	S107, T135, Y189, S320, S495, S654, S671, T834	
	NLRP3	NLR family, pyrin domain containing 3		13	S5, Y13, S161, S163, S198, T233, S295, S334, S387, S436, S728, Y861, S975	See Table 2
	NLRP4	NLR family, pyrin domain containing 4		1	S605	
	NLRP5	NLR family, pyrin domain containing 5		7	S16, T185, S191, S331[*], S642, S1062, S1064	PRKCE[*]
	NLRP6	NLR family, pyrin domain containing 6		2	Y72, S749	
	NLRP7	NLR family, pyrin domain containing 7		12	T2, S3, T61, T81, S285, Y318, S460, T732, T734, T737, Y810, T849	
	NLRP8	NLR family, pyrin domain containing 8		2	S544, S935	
	NLRP9	NLR family, pyrin domain containing 9		5	S269, S270, S691, Y693, S952	
	NLRP10	NLR family, pyrin domain containing 10		9	T36, S38, S268, Y323, Y327, T377, T511, S639, T640	
	NLRP11	NLR family, pyrin domain containing 11		4	S439, Y446, S940, S943	
	NLRP12	NLR family, pyrin domain containing 12		3	S224, S727, S979	
	NLRP13	NLR family, pyrin domain containing 13		2	T243, T358	
	NLRP14	NLR family, pyrin domain containing 14		8	T101, S302, T310, T311, S312, Y443, T1022, S1040	
NLRX	NLRX1	NLR family member X1		9	S34, S285, S293, T428, T514, S600, S697, S943, T947	

PYD NACHT LRR LRR NT/CT CARD FIIND AD BIR (x3)

Human / Murine Residue	Domain	Reference LTP / HTP	Result of Phosphorylation	Kinase	Phosphatase
S5 / S3	PYD	LTP [47]	Inhibitory, conformational change	Unknown	PP2A
Y13 / Y11	PYD	LTP [43]	Unknown	Unknown	Unknown
S161 / S157	–	LTP [47]	No effect	Unknown	Unknown
S163 / D159	–	LTP [43]	Unknown/No effect	Unknown	Unknown
S198 / S194	–	LTP [43]	Activating, conformational change, ubiquitination	JNK1	Unknown
T233 / T229	NACHT	HTP [48]	Unknown	Unknown	Unknown
S295 / S291	NACHT	LTP [46]	Activating	PKD	Unknown
		LTP [51,52]	Inhibitory	PKA	Unknown
S334 / S330	NACHT	LTP [43]	No effect	Unknown	Unknown
S387 / S383	NACHT	HTP [49]	Unknown	Unknown	Unknown
S436 / S432	NACHT	HTP [50]	Unknown	Unknown	Unknown
S728 / S725	–	LTP [43,47]	Unknown	Unknown	Unknown
Y861 / Y858	LRR	LTP [42]	Inhibitory	Unknown	PTPN22
S975 / N972	LRR	LTP [43]	No effect with priming	Unknown	Unknown