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Review

Sensing disease and danger: A survey of vertebrate PRRs and their origins

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ABSTRACT

A key facet of the innate immune response lays in its ability to recognize and respond to invading microorganisms and cellular disturbances. Through the use of germ-line encoded PRRs, the innate immune system is capable of detecting invariant pathogen motifs termed pathogen-associated molecular patterns (PAMPS) that are distinct from host encoded proteins or products released from dying cells, which are known as damage-associated molecular patterns (DAMPs). PAMPs and DAMPs include both protein and nucleic acids for the detection and response to pathogens and metabolic "danger" signals. This is by far one of the most active areas of research as recent studies have shown retinoic acid inducible gene 1 (RIG1)-like receptors (RLRs), the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs) and Toll-like receptors (TLRs) and the recently described AIM-like receptors (ALRs) are responsible for initiating interferon production or the assembly and activation of the inflammasome, ultimately resulting in the release of bioactive IL-1 family members. Overall, the vertebrate PRR recognition machinery consists of seven domains (e.g., Death, NACHT, CARD, TIR, LRR, PYD, helicase), most of which can be traced to the very origins of the deuterostomes. This review is intended to provide an overview of the basic components that are used by vertebrates to detect and respond to pathogens, with an emphasis on these receptors in fish as well as a brief note on their likely origins.

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1. Introduction—an overview of PRRs

The late Charlie Janeway is a father in the field of pattern recognition. More than 20 years ago, in 1989, Janeway predicted that pattern recognition receptors would evolve into a major focus for investigators of the innate and adaptive immune responses of jawed vertebrates (Janeway, 1989; Medzhitov, 2009), roughly 100 years after Metchnikoff described the protective capacity of phagocytic cells against microbial infection and thus the power of comparative biological studies (Metchnikoff, 1891). Janeway was absolutely correct and it is now known that the evolutionary origins of the mechanisms and strategies of pattern recognition utilized by innate immunity predate the emergence of gnathostomes.

Vertebrates have evolved a vast array of both extracellular and intracellular pathogen recognition receptors (PRRs) for detecting and responding to pathogen-associated molecular patterns (PAMPs) of viruses and microbes—also called microbe-associated

Abbreviations: NLR, NOD-like receptors; TLR, Toll-like receptors; PAMPS, pathogen associated molecular patterns; PRR, pathogen recognition receptors; CARD, caspase recruitment domain; ASC, apoptosis-associated speck-like protein; IL1b, interleukin 1 b; LRR, leucine-rich repeat.

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Table 1
Typical protein domains of pattern recognition receptors.

	TLR	NLR	RLR	ALR
Effector/signaling domains				
TIR	X			
CARD		X	X[a]	
PYD		X		X
Other		X		
Regulatory domains				
NACHT		X		
DExD			X	
PAMP recognition domains				
LRR	X	X		
Helicase			X	
RD			X	
HIN				X

[a] LGP2, a RLR member, lacks an effector (CARD) domain.

molecular patterns (MAMPs)—or to danger-associated molecular patterns (DAMPs) of endogenous molecules released by damaged or stressed host cells. Bacterial PAMPs include components of the bacterial cell wall (e.g. LPS and peptidoglycan), flagellin and DNA or RNA structures that are unique to bacteria, while viral PAMPs for the most part entail unique nucleic acids, including both double-stranded and single stranded non-capped RNA. Fungal PAMPs are associated with early germ tube and hyphal forms of *fungus* that express high levels of zymosan—a known TLR2 agonist. DAMPs (also called alarmins) include proteins such as heat shock proteins, calgranulins, and serum amyloid A, and non-protein molecules such as uric acid, ATP, potassium efflux, ROS, and heparin sulfate (Bianchi, 2007; Piccinini and Midwood, 2010).

Using a related nomenclature, germline-encoded PRRs currently are classified into five major groups: the C-type lectins (which are not addressed in this review), the well known Toll-like receptors (TLRs); retinoic acid inducible gene I (RIGI)-like receptors (RLRs); the nucleotide-binding domain, leucine-rich repeat containing proteins (NLRs; previously designated as the nucleotide binding oligomerization domain (NOD)-like receptors); and the newly classified family of HIN200/PYHIN family members that have recently been designated Absent in melanoma (AIM)-like receptors (ALRs). For the most part, TLRs, RLRs, NLRs, and ALRs are expressed by first responder cells of the immune system, including monocytes, macrophages, dendritic cells, and neutrophils. In addition, some non-immune cells express PRRs, including tissue-specific epithelial and endothelial cells as well as cells of the nervous system. The cellular location of PRRs varies: they are expressed on the cell surface (TLRs), in endosomes (TLRs) or within the cytosol (NLRs, RLRs and ALRs) of host cells.

The basic characteristics of all PRRs are the same; they all possess a protein domain for recognizing PAMPs (or DAMPs) coupled—sometimes with an intervening domain—to a protein domain that interacts with downstream signaling molecules. A limited collection of functionally analogous protein domains for both PAMP recognition and signal propagation is shared between PRRs (Table 1). Leucine-rich repeat (LRR) domains are essential for the specificity of PAMP recognition by both TLRs and NLRs. LRR are also present in the variable lymphocyte receptors of agnathan that are analogous to antibodies in fish, indicating the specificity and usage of LRR in recognizing non-self is not limited to PRR (Pancer et al., 2004, 2005). ALRs use the unique hematopoietic interferon-inducible nuclear proteins that contain a 200-amino acid repeat (HIN-200) domain(s) for PAMP recognition (Hornung et al., 2009), whereas RLRs bind PAMPs with both helicase and regulatory domains (RD) (Yoneyama and Fujita, 2008). After sensing PAMPs, PRRs deliver a signal via their effector or signaling domain. In TLRs, the effector domain is a Toll/interleukin-1 receptor (TIR) domain. However, NLRs, RLRs and ALRs use either a caspase recruitment domain (CARD) or the related PYRIN domain (PYD) to interact with other proteins.

Upon ligand recognition (PAMPs or DAMPs), all of these receptors depend upon the interaction of specific bridging proteins to initiate a cascade that results in signaling events. In general, PRR signaling eventually activates specific transcription factors (e.g. NFκB or IRFs) and kinases (e.g. MAPK) and creates a network of cross-talk by which PRR regulate multiple host immune and pro-inflammatory genes and co-ordinate an appropriate immune response to the detected pathogen or tissue injury. As such, these innate immune receptors can no longer be viewed simply as non-specific receptors for host immunity, but rather as a highly complex, divergent and specific foundation for pathogen and danger recognition and elimination.

2. Toll-like receptors have deep evolutionary histories

First described by Jules Hoffmann and colleagues as a key component in responding to fungal infection in insects (Lemaitre et al., 1996), TLRs are now likely the most extensively studied group of PRRs in both vertebrates and invertebrates. TLRs comprise a multigene family in mammals; there are 10 human TLRs (TLR1–10) and 12 murine TLRs (TLR1–9 and 11–13) that each have a different PAMP specificity (Table 2). The evolutionary history of the TLR family suggests that this holds true for non-mammalian vertebrates as well (summarized in Kasamatsu et al., 2010). There are about 10 distinct TLR genes in birds, including two forms each of TLRs 1 and 2, and one each of TLRs 3–5, and 7. Three TLRs found in birds differ from those described in mammals (TLR15, 16, and 21; TLR21 has two forms) (Keestra et al., 2007; Temperley et al., 2008). Amphibians possess approximately 20 TLRs that, like birds, often contain duplicated forms (TLRs 2, 6, 8, and 14) (Ishii et al., 2007a).

Teleost fish encode orthologs to the vast majority of TLRs found in the above tetrapods (e.g. mammals, birds, amphibians) plus additional TLRs (TLR20–TLR23) for a total of 17 distinct TLRs (Roach et al., 2005; Huang et al., 2008; Oshiumi et al., 2008; Rebl et al., 2010) (Table 2). Of these, several are direct structural orthologs of mammalian TLRs (e.g. TLRs 1–5, 7–9, 12–13) while others have no predicted mammalian orthologs (e.g. TLRs 14 and 18–23) and may be represented by multiple paralogs (e.g. TLR20). Curiously, cyprinids possess TLR4-like genes, whereas TLR4 appears absent from other teleost species such as fugu, *Tetraodon*, and stickleback. On this point it should be noted that the zebrafish TLR4 sequences are quite divergent and functional data indicates that the extracellular region (LRR) of zebrafish TLR4 cannot sense LPS (Sullivan et al., 2009). Lampreys, representing agnatha, possess similar number of TLRs (16 models) to teleost fish—the lamprey TLRs include unique genes (e.g. TLR24a–24d), and those similar to prototypical mammalian TLRs (TLR3, 5, and 7/8) as well as to the non-mammalian TLRs (e.g. TLR21 and 22) (Ishii et al., 2007b; Kasamatsu et al., 2010).

TLRs are an ancient family of PRR—even non-vertebrate genomes encode multiple TLRs. On this note, the amphioxus genome has 28 predicted TLR complete gene models (>70 if partial models are included) composed of combinations of LRR, TM and TIR domains, whereas there are over 200 TLRs in the sea urchin, at least 8 unique TLRs in Drosophila (Huang et al., 2008; Messier-Solek et al., 2010) but only a single TLR in the "reductionist genome" of the short lived pelagic tunicate, *Oikopleura* (Denoeud et al., 2010). The expanded repertoire and diversity of TLRs in cephalochordates and the long-lived echinoderms likely reflects a gene family that evolved rapidly to cope with either rapidly evolving microbes or a greatly expanded array of pathogens found in aquatic environments (Hibino et al., 2006; Huang et al., 2008; Messier-Solek et al., 2010; Rast et al., 2006).

To date, most knowledge of TLR expression and function is based on mammalian TLRs. Some TLRs are expressed on the cell surface

Table 2
TLRs identified in distinct vertebrate taxa to date.

Group	TLR	Ligand	Mammal	Bird	Amphibian	Teleost	Agnathan
TLR1	TLR1	(diac)lipopeptide	X	X	X	X	
	TLR2	PGN	X	X	X	X	
	TLR6	(triac)lipopeptide	X	X	X		
	TLR10	Lipopeptides(?)	X				
	TLR14	nk			X	X	X
	TLR15	Unknown bacterial		X			
	TLR16	Lipopeptides		X			
	TLR18	nk				X	
	TLR24	nk					X
TLR3	TLR3	Short dsRNA	X	X	X	X	X
TLR4	TLR4	LPS/host proteins	X	X	X[a]	X[b]	
TLR5	TLR5	Flagellin	X	X	X	X	X
TLR7	TLR7	ssRNA	X	X	X	X	X
	TLR8	ssRNA	X		X	X	
	TLR9	CpG	X		X	X	
TLR11	TLR11	Profilin	X				
	TLR12	nk	X		X	X	
	TLR13	nk	X		X	X	
	TLR20	nk				X	
	TLR21	CpG		X	X	X	X
	TLR22	Long dsRNA			X	X	X
	TLR23	nk				X	
Unknown	TLR19	nk				X	

[a] Denotes that a highly divergent TLR4 receptor was identified (Ishii et al., 2007a,b). Xenopus are non-responsive to LPS (Morales et al., 2003; Robert, 2003).
[b] Zebrafish TLR4 does not respond to LPS—likely due to lack of LRR engagement of ligand (Sullivan et al., 2009). NK-not known.

(e.g. human TLR1, 2, 4, 5, 6, and 10), while others are expressed in endosomes (e.g. human TLR3, 7, 8, and 9). In each case, the TIR moiety resides in the cell cytoplasm while the LRR domain is positioned to detect either extracellular PAMP (outside of the cell) or during sampling within endosomes. Many TLR function as homodimers, although TLR2 heterodimerizes with TLR1 or TLR6 to provide different PAMP specificity (Takeuchi et al., 2002; Triantafilou et al., 2006). TLR ligand specificity is due to different combinations of LRRs. Upon engagement of their LRRs with ligand (e.g. flagellin, dsRNA, LPS, zymosan), TLR engage specific adaptor molecules using their cytoplasmic TIR domains; these adaptors trigger inflammatory cascades that lead to the production of cytokines and type I interferons, which are critical for innate responses such as phagocytosis and respiratory burst, and for activation of the adaptive immune system.

Divergent TLRs require divergent downstream binding partners—a handful of these proteins have been described for TLRs in mammals: myeloid differentiation primary response gene-88 (MyD88); TIR-containing adaptor protein (TIRAP), also called MyD88 adaptor-like (MAL); TIR-containing adaptor molecule-1 (TICAM1), which is also called TIR-containing adaptor protein inducing IFNβ (TRIF); TIR-containing adaptor molecule-2 (TICAM2), which is also called TRIF-related adaptor molecule (TRAM); and sterile α-and armadillo-motif-containing protein (SARM) [reviewed by O'Neill and Bowie (2007)]. All mammalian TLRs, except TLR3, interact with MyD88 to activate NF-κB, MAP kinase, or IRF signaling pathways; many also interact with a second adaptor, while TLR4 interacts with several adaptors. TIRAP is used as a second adaptor by TLR2 and 4 in MyD88-independent activation of NF-κB (Henneke and Golenbock, 2001). TLR3 solely uses TICAM1—a further adaptor molecule also utilized by TLR4 to initiate IRF signaling—to induce production of type I IFNs via IRF3 and IRF7 signaling. TICAM2 is also exclusively used by TLR4 for late activation of NF-κB and IRF3. SARM is an adaptor protein that negatively regulates TLR activity by inhibiting signaling by TICAM1. Many TLR signaling proteins (including MyD88, TIRAP, TICAM1 and SARM, but not TICAM2) are structurally and functionally conserved in teleost fish, although the fine details within the signaling pathways vary from the mammalian paradigm (Purcell et al., 2006; Stein et al., 2007; Sullivan et al., 2007; Fan et al., 2008). For instance, the interaction between zebrafish TICAM1 and TLR3 is conserved but resulting activation of type I IFN is independent of IRF3 or 7 (Sullivan et al., 2007). Thus, TLR signaling developed early, but has also adapted to use different pathways in distinct vertebrates, further highlighting the plasticity of the immune system.

3. RIG-I-like receptors: mediators or virus recognition

Helicases of the RLR family are crucial for inducing an antiviral state. Representative RLRs include retinoic acid inducible gene protein I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which are largely responsible for recognizing cytosolic, virus-derived RNA and subsequently inducing the production of interferons (Takeuchi and Akira, 2008). Remarkably, RIG-I is capable of discriminating vertebrate RNA (host) from viral RNA based upon the chemical structure at the 5′ end of RNA: host mRNA has a capped 5′ end while viral single stranded RNA (ssRNA) detected by RIG-I has an uncapped 5′ triphosphate group (Hornung et al., 2006). RIG-I can also recognize short double stranded RNA (dsRNA). MDA5, in contrast, recognizes long dsRNA (Takahasi et al., 2009).

The structures of MDA5 and RIG-I are very similar. Each possesses one or two CARD domains at their N-terminus, followed by a DExD/H or closely related type III restriction enzyme (Res III) domain, a helicase domain, and a C-terminal regulatory domain (RD) (Yoneyama and Fujita, 2007, 2008; Yoneyama et al., 2005; Zou et al., 2009) (Fig. 1). Specific amino acid differences in the RDs of RIG-I and MDA5 provides their ability to discriminate and bind viral derived nucleic acids (Yoneyama and Fujita, 2008) (Kato et al., 2008; Pichlmair et al., 2009; Takeuchi and Akira, 2008). Once the ligand is bound by the RD, a conformation shift is thought to orientate the CARD domain(s) of the RLR to interact with the CARD domain of an adaptor protein—the mitochondrial antiviral signaling protein, MAVS (also known as Cardif, VISA and IPS-1) is the

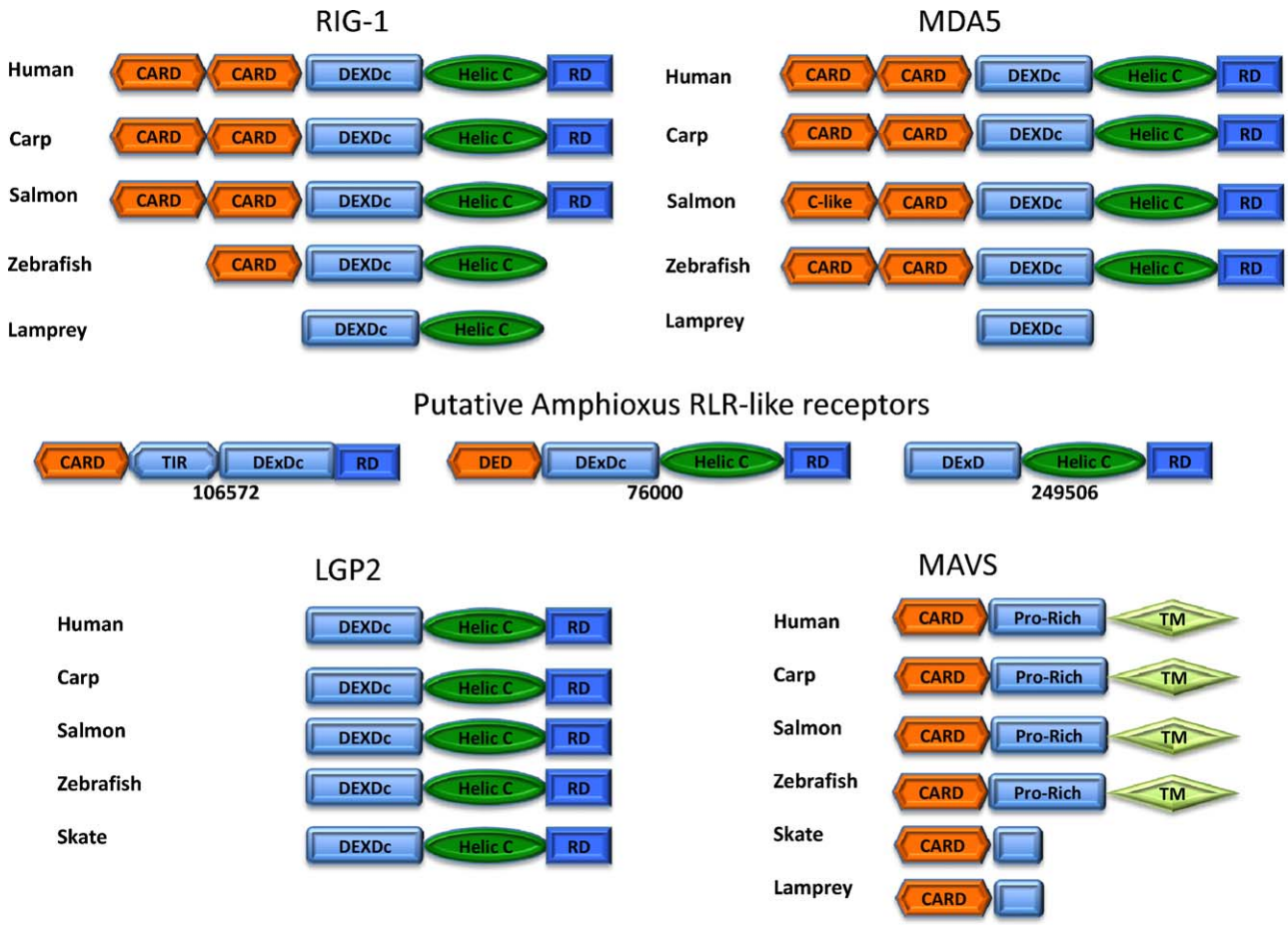

Fig. 1. Domain architecture of RLRs and MAVS in various vertebrates. Sequences were obtained either from previous publications (Huang et al., 2008; Zou et al., 2009; Biacchesi et al., 2009) or through tBLASTn or Ensembl analysis. The zebrafish RIG-I sequence (ENSDARP00000058175) previously localized to Zf Chr23 has been reassigned to NA266 in the new assembly. Additional putative zebrafish RIG-I ESTs DY552702 and CD594255 are found within ENSDARP00000072958. Putative lamprey exons containing the DEXDc domain were found on contig_50584—this domain showed 50% identity over 196 amino acids to teleost RIG-I. RLR sequences encoding the elasmobranch DEXDc/HELICc/RD domain model were found in skate (FF601439 and GE766377). Additional elasmobranch RLR partial sequences were also present in the dogfish ESTs (e.g. EE049339 or FF600461). Domains were determined using SMART (http://smart.embl-heidelberg.de), InterProScan (www.ebi.ac.uk/Tools/InterProScan) and the TMpred server (www.ch.embnet.org). *Abbreviations*: CARD: caspase recruitment domain, DEXDc: DEAD-like C-terminal helicase domain, HelicC: Helicase conserved C-terminal domain, RD: RIG-I repression/regulatory domain, TM: transmembrane domain, Pro-Rich: proline rich domain and TIR: TOLL/IL-1R domain.

adaptor protein for both RLRs. This interaction is required for type I interferon induction (Sun et al., 2006). The RIG-I/MDA5 signaling pathway is strictly regulated to provide tight control of IFN production. One level of regulation for this pathway is accomplished by a third member of the RLRs: laboratory of genetics and physiology 2 (LGP2) (Komuro et al., 2008). LGP2 is structurally related to both RIG-I and MDA5; it contains both helicase and RD domains but lacks a CARD (Fig. 1) (Yoneyama et al., 2005; Rothenfusser et al., 2005). Initially LGP2 was believed to negatively regulate RLR signaling via sequestration of dsRNA, complexing with MAVS, or by directly binding RIG-I (Komuro et al., 2008; Komuro and Horvath, 2006), although recent studies suggests that LGP2 can also potentiate IFN production in response to viral infection (Satoh et al., 2010)—hence the true role of LGP2 as a positive or negative regulator of anti-viral immunity remains to be clarified.

In depth gene synteny analyses have identified RIG-I and MDA5 gene orthologs in avian, amphibian and teleost species (Zou et al., 2009; Barber et al., 2010). Interestingly, RIG-I appears to exist in some but not all teleost genomes suggesting that they were either lost from particular fish genomes or have diverged to a level that they are no longer recognizable (Zou et al., 2009). For instance LPG2 and MDA5 appear common to all teleost genomes whereas teleost RIG-I has been identified only for cyprinids and salmonids (Biacchesi et al., 2009; Zou et al., 2009; Huang et al., 2010). Functional characterization confirms similar activity of fish RLR. Teleost MDA5 and LGP2 are both upregulated in a rapid but transient manner in lymphoid tissues during virus infection in grass carp indicating a potential involvement in the early anti-viral response (Su et al., 2010; Huang et al., 2010) and over-expression of either full-length teleost RIG-I or the RIG-I CARD domain alone leads to significant induction of an antiviral state as measured by plaque reduction assays and the induction of IFN stimulated genes including the RLRs and IFN (Biacchesi et al., 2009). On a related evolutionary note, avian RIG-I also participates in an anti-viral state against influenza via detection of viral RNA (Barber et al., 2010)—thus further implying that the functional role of RIG-I is conserved from fish through mammals.

Based upon the presence of RLRs in fish, its not surprising that a number of groups have also recently reported the presence of MAVS orthologs in several species of fish, including partial sequences from elasmobranchs and agnathans (Biacchesi et al., 2009; Lauksund et al., 2009; Simora et al., 2010). Fish and mammalian MAVS have the same domain structure—with an N-terminal CARD and a C-terminal transmembrane domain—and appear to have conserved function as mammalian MAVS (Biacchesi et al., 2009). Over-expression of teleost MAVS protects cells from infection by both DNA and RNA viruses by inducing interferon stimulated genes, such as IRF3, Mx, and Vig-1, as well as type I IFN, confirming a

role for this protein in anti-viral pathways (Biacchesi et al., 2009; Lauksund et al., 2009; Simora et al., 2010) (Fig. 1). In addition, teleost MAVS proteins are associated with mitochondria, like mammalian MAVS (Biacchesi et al., 2009), raising one question that has yet to be addressed: why are the MAVS associated with the mitochondria? Aside from being a scaffold for RLR-MAVS interactions, it could be that MAVS also signal to the mitochondria to alter energy production for the immune response itself as this is an energy dependent process (Scott, 2010). This has not been addressed but considering that MAVS have been tethered to the mitochondria throughout vertebrate evolution, there must be a benefit for the host that we do not yet fully appreciate.

Confirming RLRs stem from an ancient family of PRRs with origins in invertebrates, ancestral molecules with conserved structure and genomic architecture relative to both RIG-I and MDA5 were also identified in sea anemone and amphioxus (Huang et al., 2008; Zou et al., 2009). Based upon the putative RLR models found in amphioxus, we re-scanned the amphioxus genome further for the presence of the RD of RIG-I and found BRAFLDRAFT_106572, a gene model encoding characteristics of both RLRs and TLRs as it possesses the following domain structure: CARD-TIR-DExDc-RD (Fig. 1). The presence of these features strongly suggests that the amphioxus protein plays a role in immunity. Several additional paralogs (BRAFLDRAFT_76000, 249506, 249510, 235564, and 93024) with structures analogous to RIG-I/MDA5/LPG2 were also identified in the amphioxus genome; of note, these all possess a CXXC cysteine motif within the RD domain that is needed for ligand engagement (Fig. 1). These models are distinct from amphioxus DICER (BRAFLDRAFT_202604), a related homolog of RLRs involved in RNA silencing events, which lack an RD. In addition several models consistent with LGP2 were also found, further implying that the RLR family and likely functionality were set prior to the emergence of the vertebrates. This, coupled with potential MAVS-like homologs found in agnathans (Biacchesi et al., 2009) and potential RLR-like adaptors sequences in invertebrates (sea urchins and amphioxus) (Hibino et al., 2006; Huang et al., 2008; Messier-Solek et al., 2010; Zou et al., 2009), indicates the origins of the RLR system of viral detection predate the emergence of the gnathostomes and likely that of all deuterostomes.

4. NOD-like receptors

The NLR family of intracellular sensors has emerged as a crucial component of the innate immune response owing to their roles in inducing and processing IL-1 family members. Mammalian NLRs comprise five gene categories designated NLRA, NLRB, NLRC, NLRP and NLRX, which each share three main domains and the same overall domain organization. All NLRs contain a central NACHT domain, variable number of C-terminal leucine-rich repeats (LRRs), and an N-terminal effector domain that varies between gene categories (Fig. 2): NLRC (NLR family, CARD domain containing) genes (NLRC1–5) have an N-terminal CARD; NLRP (NLR family, pyrin domain containing, aka NALPs-NACHT, leucine-rich repeat and PYD containing protein) genes (NLRP1–14) have an N-terminal PYD; NLRA (the MHC class II transactivator; CIITA) possesses either the MHCIIACTVATR domain (AD) or a CARD-like domain prior to the AD domain in some splice variants only; the effector domain of NLRB (NLR family apoptotic inhibitory protein; NAIP; NLRB1) is composed of baculoviral IAP repeats (BIR); and the solitary NLRX gene (NLRX1) has a unique effector domain. The central NACHT domain is unique to NLRs and is critical for their oligomerization, while the C-terminal LRR domain, as in TLRs, serves to recognize PAMPs and associated danger signals. Ligand sensing by the LRR results in NACHT oligomerization, which shifts the receptor conformation such that the N-terminal effector domains are accessible for interacting with signaling molecules or molecular adaptors.

The distinct effector domains of NLRs allow specificity of signaling. Two NLRCs, NLRC1 (NOD1) and NLRC2 (NOD2) interact with RIP2 kinase to induce pro-inflammatory molecules—including members of the IL-1β family of cytokines—via activation of NFκB upon sensing bacterially derived peptidoglycan (summarized in Fritz et al., 2006; Strober et al., 2006) (Fig. 3). NLRPs and some NLRCs, interact with inflammatory caspases (caspase-1 and caspases-5), either directly or with the assistance of the bridging protein apoptosis-associated speck-like protein containing a CARD (ASC, also called PYCARD). By these interactions, these NLRs form a multiprotein complex that is termed the inflammasome, which consists of one or more NLRs, the adaptor protein ASC and inflammatory caspases. In the inflammasome, inflammatory caspases are activated and process IL-1β, IL-18, and IL-33 pro-cytokines (generated by TLR or NLRC1/NLRC2 activation and recruited by inflammatory caspase)—the inflammasome is therefore a central component of inflammation in vertebrates (Fig. 3).

ASC serves as an adaptor protein to allow interactions between NLRPs and caspase-1 and formation of the inflammasome. ASC also directly interacts with the apoptotic protein pyrin (Richards et al., 2001), showing its involvement in apoptosis. In resting cells, ASC localizes to the nucleus but is quickly redistributed to the cytoplasm upon infection where it assists NLRs or ALRs and caspase-1 to form aggregates that can be visualized as "specks" (Bryan et al., 2009; Jones et al., 2010); these specks represent the inflammasome. Formation of the inflammasome is a highly regulated process; only one speck forms per macrophage, typically within hours of infection (Bryan et al., 2009; Jones et al., 2010), and truncated alternative splice variants of ASC competitively bind NLRs, ALRs, and caspase-1 to inhibit inflammasome formation (Bryan et al., 2010; Matsushita et al., 2009).

Many of the NLR genes discovered in mammals are preserved in non-mammalian vertebrates including fish (Fig. 2), although teleost fish possess an additional unique group of NLRs (Stein et al., 2007; Laing et al., 2008). NLRCs and NLRPs are two major subfamilies of NLRs. Mammalian NLRC1 and 2 (NOD1 and 2) discriminate bacterial pathogens via detection of bacterial cell wall components; they recognize diaminopimelic acid (DAP, restricted to gram negative bacteria) and muramyl dipeptide (MDP, found in gram negative and positive bacteria) respectively using their distinct LRRs. Upon ligand interaction, these NLCs associate with RIP2 kinase via CARD-CARD interactions to induce IL-1β synthesis, as described above. Both NLRC1 and NLRC2 are conserved in fish. Teleost NLRC1 and 2 (designated either NOD1 and NOD2, or NLR-A1 and NLR-A2) have the same structure as their mammalian orthologs and their transcripts are upregulated during bacterial and viral infection, or stimulation with polyI:C (Chang et al., 2010; Chen et al., 2010; Sha et al., 2009). In addition, overexpression of the CARD domain of trout NOD2 significantly induces pro-inflammatory genes, including IL-1β, presumably through interaction with RIP2 kinase (Chang et al., 2010). Since an orphan CARD domain inhibits NFκB signaling when overexpressed in zebrafish, the induction of a pro-inflammatory response appears to be specific to the NLR CARD domains in teleost fish (Chang et al., 2010). NLRC3 (NOD3) is well conserved in the vertebrates and is thought to play a role in modulating T cell responsiveness—the functional role of NLRC3 has received little attention but interestingly, the NACHT domain of NOD3 is well conserved amongst additional NLRC genes in fish.

Of the NLRs that function via the inflammasome, NLRP3 is the most extensively studied. It responds to a diverse range of PAMPs from microbes and fungal pathogens including LPS, peptidoglycan, alum, beta-glucan, silica and microbial nucleic acids as well as reacting to DAMPs (ROS, ATP and mono-sodium urate, injury—hyaluronan) (recently summarized in (Brodsky and Monack, 2009; Martinon et al., 2009; Tschopp and Schroder, 2010) (Fig. 3). The wide repertoire of ligands for NLRP3 may reflect its

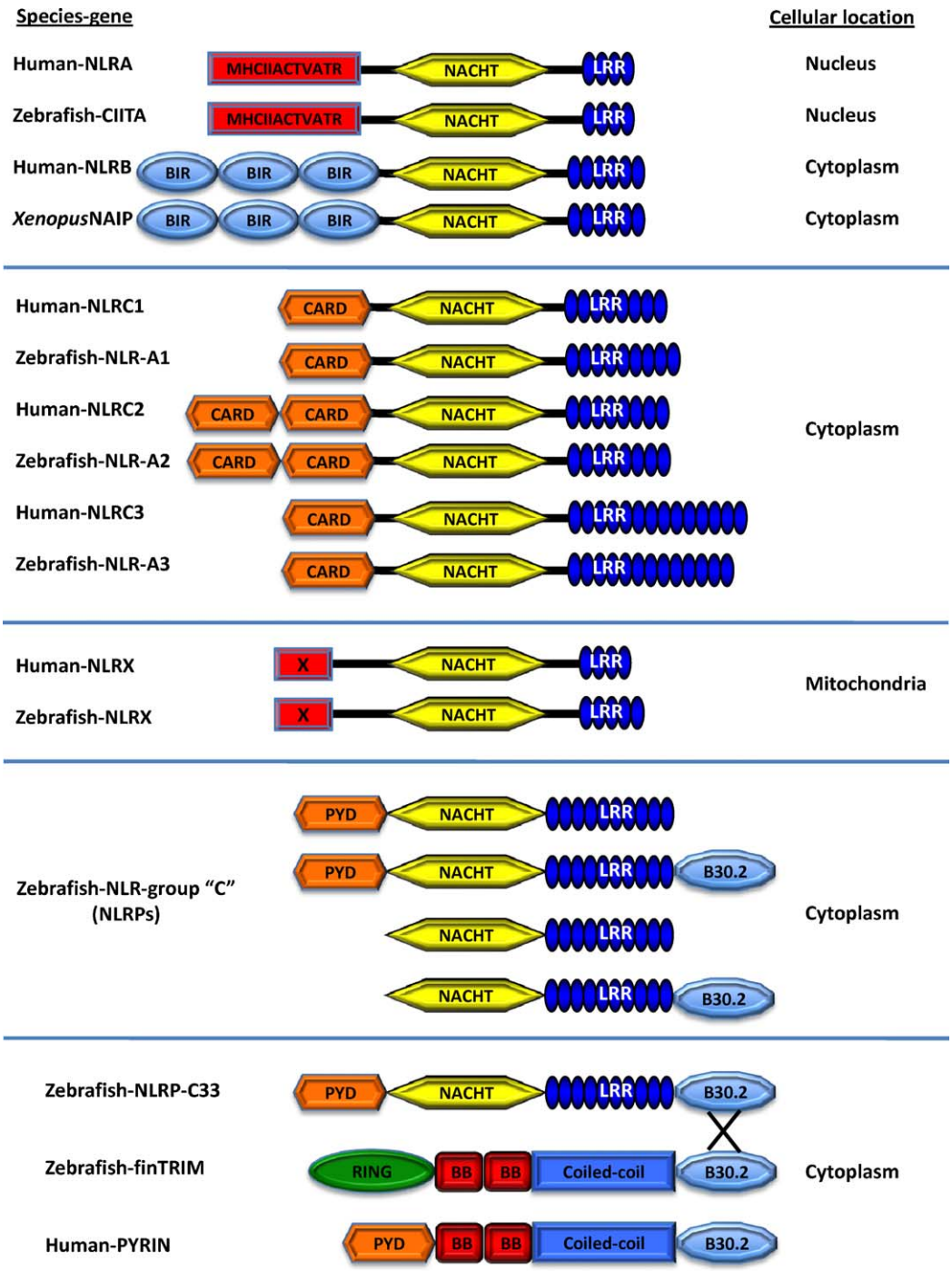

Fig. 2. Schematic representation of NLRs in mammals, amphibians and teleost fish. Sequence accession numbers can be found in Stein et al. (2007), Laing et al. (2008) and van der Aa et al. (2009) with the exception of human PYRIN (aka MEFV, TRIM20) whose accession number is NM_000243. Cellular localization was predicted using pSORT (www.psort.org or http://wolfpsort.org). Putative domain shuffling (B30.2 domain) between fish NLRs and finTrims as suggested by Du Pasquier (2009) are signified by an X. *Abbreviations*: BIR: baculovirus inhibitor of apoptosis protein repeat, BB: B box domain, PYD: pyrin domain (aka PAAD_DAPIN), CARD: caspase recruitment domain, LRR: leucine rich repeats, NACHT: nucleotide binding/oligomerization domain, MHCIIACTVATR: MHC Class II transactivator motif 1–11 and RING: RING-type zinc finger domain.

response to universal downstream molecules such as reactive oxygen species (ROS) or ion efflux generated in response to PAMPS and DAMPs and is likely linked to P2X7 receptor activation and ROS production by the mitochondria—further highlighting the role of the mitochondria in inflammatory processes. NLRC4 forms an inflammasome with caspase-1 and is responsible for recognizing diverse bacterial pathogens including *Listeria*, *Mycobacteria* and *Salmonella*. The PAMP recognized by NLRC4 is a conserved motif on cytosolic flagellin and the basal body rod component of the bacterial type-III secretion system (Miao et al., 2010). NLRP1 is thought to sense muramyl dipeptide (MDP) and is structurally unique among the NLRPs in that addition to an N-terminal PYD domain, it contains a C-terminal CARD domain and in conjunction with ASC forms an inflammasome containing caspase-1 and 5. In mice, NLRP1b recognizes anthrax lethal toxin resulting in NLRP1 inflammasome activation—one interesting aspect here is that NOD2 (NLRC2) also participates in the NLRP1 inflammasome (Hsu et al., 2008), thus playing roles not only for IL-1β transcription but processing as well. Despite a plethora of studies describing the three best-studied inflammasomes (NLCR4, NLRP3 and NLRP1) there is little known

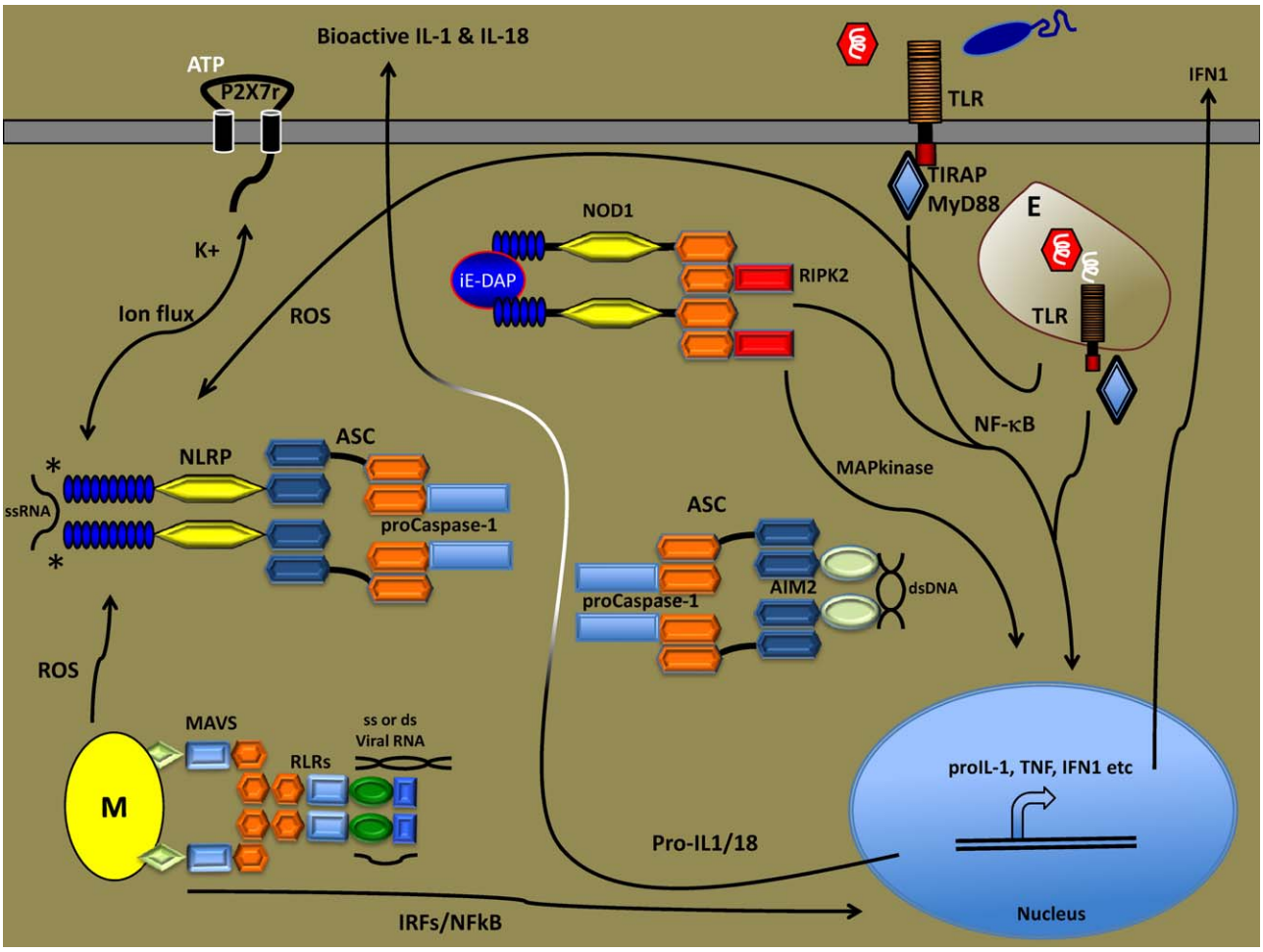

Fig. 3. Simplified overview of the PRR signaling pathways for IFN and IL-1β. TLRs (LRR-TM-TIR) are expressed at the cell surface and within endosomes (**E**: TLR3, 7–9) where they interact with a wide variety microbial PAMPs to transmit signals via specific adaptors (e.g. MyD88, TRIF) to induce pro-inflammatory gene (e.g. IL-1β) and IFN expression. NOD1 and 2 detect breakdown products of bacterial peptidoglycans (MDP and ieDAP) and activate NFκB via RIP2K and MAP kinases leading to induction of pro-IL-1β and general pro-inflammatory responses. NLRP inflammasomes (NLRP1, NLRP3 and NLRC4) are the best-characterized activators of active caspase-1, which processes pro-IL1β into its biologically active form. NLRP3 has been shown to sense a variety of microbial PAMPs and cellular DAMPs (including reactive oxygen species [ROS] and potassium efflux, the latter involving the purinergic receptor P2X7 and ATP). The ALRs, including AIM2 sense dsDNA, and form a caspase-1 inflammasome via interaction with ASC. Thus TLRs and NLRs both contribute to IL-1β production but NLRP or ALR inflammasomes are needed for processing. RLRs (e.g. RIG-I, MDA5: CARD-CARD-DEXDc-HELICc-RD) can differentiate ss or ds viral RNA and transmit signal via interaction with RLR adaptor, MAVS complexed to the mitochondrial (**M**) membrane; these interactions transmit the signal through both NFκB and specific interferon regulatory proteins (IRF3 and 7) for the production of IFNs. The white portion of the "IL-1/I8" arrow indicates processing of pro-IL-1/18 by the caspase-1 p20 subunit ("activated caspase") from the NLRP and AIM inflammasomes.

about the roles of the other NLRP proteins and their roles for immunity.

The NLRP family in bony fish is a conundrum. Multiple NACHT domains, which are phylogenetically related to the NACHT domains of mammalian NLRP are evident in the zebrafish genome indicating these genes multiplied in fish just as in mammals although they do not encode recognizable LRR or PYD domains. However, teleostei also possess an expanded family of PYD containing NLRs (termed NLR-group C in zebrafish, >70 members) (Laing et al., 2008; Stein et al., 2007). These novel fish NLRPs possess an N-terminal PYD coupled to NACHT and LRR domains and some curiously possess additional C-terminal B30.2 domains (PRY/SPRY). The presence of NLRPs containing B30.2 domains appears unique to teleost fish. We and others (van der Aa et al., 2009) propose that this B30.2 domain may interact with ligand in a manner akin to similar domains in TRIM proteins (Diaz-Griffero et al., 2008; McEwan et al., 2009) since it shares such high sequence identity (~60%) to the B30.2 domain found in finTRIMs. FinTRIMs are a highly diversified, multigene family of TRIM proteins restricted to teleost fish with a likely role in anti-viral immune function (van der Aa et al., 2009). Possible exon shuffling occurred to share B30.2 domains between the finTRIMs and the unique group of zebrafish NLRPs (bottom Fig. 2), an idea supported by the genomic locations of finTRIMs and NLRs (Du Pasquier, 2009). Alternatively, the teleost NLR B30.2 domain may have evolved to regulate inflammation in a manner analogous to the pyrin protein (alias TRIM20; encoded by the Mediterranean fever gene, MEFV), which has an N-terminal PYD and a C-terminal B30.2, and controls innate immunity by competitively binding ASC with its PYD or caspase-1 with its B30.2 domain (Chae et al., 2006). Further complicating this family is the fact that some members have a novel N-terminal effector domain instead of the PYD that likely provides additional downstream effector specificities; these latter proteins, of course, do not fit the mammalian definition of an NLRP. The teleost NLRPs (zebrafish NLR group C) have diverse LRRs with the potential for recognizing many ligands, further adding to the potential complexity of these innate immune receptors. However, similar to the majority of mammalian NLRPs, the ligand specificity and functional roles of the fish NLRPs have yet to be defined.

NRLA/CIITA orthologs were identified in fish (Laing et al., 2008; Stein et al., 2007), amphibian, and avian species. The structure (Fig. 2) for mammalian NLRA comprises the three domains MHCIIACTVATR-NACHT-LRR whereby the N-terminal

MHCIIACTVATR domain overlaps with the NACHT domain and possesses nuclear localization and export signals while the LRRs perform a novel role of regulating nuclear import and export (Camacho-Carvajal et al., 2004). Fish models for NLRA (MHCIIACTVATR-NACHT-LRR) in the genomes of *Tetraodon*, fugu, stickleback and zebrafish were all predicted (pSORT/SMART analysis) to have nuclear localization potential like mammalian NLRA—suggestive of a conserved function (Fig. 2). Our previous study failed to identify definite NLRB1/NAIP genes in fish genomes; even our additional search using the updated zebrafish draft (Zv8) could not identify appropriate gene models with the structure of NRLB1 (3XBIR-NACHT-LRR). However, baculoviral IAP repeat containing protein (BIRC)2 and BIRC3 orthologs (BIR-BIR-BIR-CARD-RING)—likely regulators of apoptosis—were present in all fish genome databases indicating the presence of a triplicated BIR domain during early vertebrate evolution that, apparently, is not associated with NACHT. In contrast, amphibians (*Xenopus tropicalis*, XP_0022934878, ENSEMBL; NCBI: NP_001072671.1) and reptiles (*Anolis carolinensis*, ENSACP00000009897) do possess NLRB1-like genes suggesting that NLRB1 evolved after bony fish split from tetrapods, or that this gene was lost in teleost lineages. Interestingly, the anole sequence was derived from a library from regenerating tails—tissues undergoing apoptotic events—suggesting its role in controlling apoptosis is retained.

One of the more interesting members of the NLRs is NLRX. The N-terminal region of human NLRX contains a predicted mitochondrial targeting sequence and indeed localizes to the mitochondria. Mitochondrial-associated expression of NLRX is directly involved in regulating anti-viral immunity mediated through the RLR adaptor MAVS (Moore et al., 2008). NLRX binds MAVS preventing RLR-MAVS interactions thereby interfering with MAVS mediated induction of type I IFN and NFκB—in this instance the mitochondria would serve as a scaffold for the regulation of innate immunity. A more recent study however demonstrated that NLRX actually translocates into the mitochondrial matrix itself and was found to increase NFκB and Jun kinase signaling by enhancing ROS production from the mitochondria (Arnoult et al., 2009; Tattoli et al., 2008). Interestingly, bioinformatic inspection of zebrafish NLRX predicts (Fig. 2) that it will also localize to the mitochondria suggesting that the role of NRLX has been maintained during vertebrate evolution while also further highlighting the role of the mitochondria not only in cell death but in innate immune responses as well.

NLRs have origins predating jawed vertebrates—a brief survey of the elasmobranch EST database and the low coverage Elephant shark genome revealed multiple, partial NLR sequences containing NACHT and NACHT-LRR models that were closely related to NLRC3 (data not shown). Based upon likely NLRs in elasmobranchs we performed a search for agnathan NLRs using the initial assembly of the sea lamprey genome, which identified in multiple NLRC genes composed of CARD-NACHT-LRR models and several NACHT-LRR models lacking identifiable N-terminal effector domains (Fig. 4). Phylogenetic analysis of the NACHT domains revealed that the majority of the NACHT domains formed a distinct clade, while two grouped well with mammalian and fish NOD1 and 2 (Fig. 5). Crossing over into the realm of the invertebrates, detailed analysis of the urchin (Hibino et al., 2006) and amphioxus (Huang et al., 2008) genomes revealed that the structure of NLRs is relatively conserved within the deuterostomes, the main exception being that all the invertebrate NLRs models lack PYD domains and instead have been suggested to utilize other death domain family members (Messier-Solek et al., 2010) (Fig. 4). These studies showed the highly diversified nature of the NLR-like proteins in invertebrates and that there are potential bridging molecules that may serve roles analogous to mammalian ASC. Moreover, detailed phylogenetic analysis indicates that all of the invertebrate NLRs, including those from Cnidarians, belong to lineage-specific groups implying that they

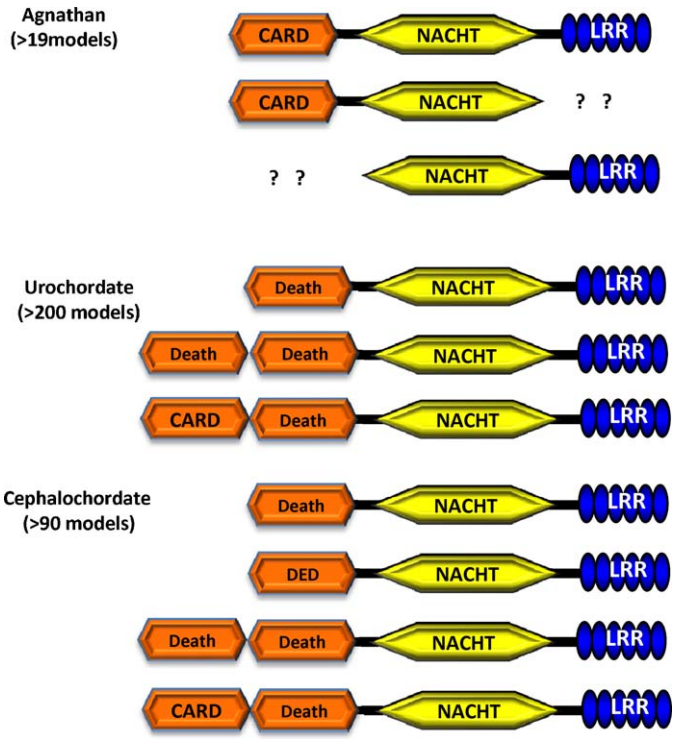

Fig. 4. Domain models for agnathan and invertebrate NLRs. Putative lamprey NLRs were identified using tBLASTn analysis of the sea lamprey genome (ENSEMBL prebuild v3.0). Lamprey NLRs identified in this study contain CARD effector domains whereas CARD and PYD domains are replaced by related death domains for the invertebrate NLR models (Rast et al., 2006; Huang et al., 2008; Messier-Solek et al., 2010). Some lamprey models lacked an N-terminal domain, hence we cannot rule out the utilization of PYD for lamprey NLRs.

are the result of several rounds of duplication of a single primordial gene (Zhang et al., 2010). The various N-terminal domains of invertebrate NLRs are proposed to fit a model where NLR domain architecture in different organisms has been guided by parallel (independent) evolution rather than convergent evolution making it difficult to assign the exact primordial architecture or identify the original effector domains (Zhang et al., 2010)—regardless, all of these studies indicate that NLRs are ancient PRRs.

Teleost fish do contain genes for ASC that appear to be functional orthologs. Recently, Sun et al. demonstrated mandarin carp ASC-GFP fusion proteins aggregate to form speck-like complexes in cellular cytoplasmic and that the majority of ASC transcription was localized to fish lymphoid tissue (Sun et al., 2008). Thus, the overall expression, structure, sub-cellular localization and interaction of ASC with the zebrafish caspase-1 homolog (Masumoto et al., 2003) are conserved within fish and mammals. Since skates and lamprey have NLR orthologs, we searched for ASC in elasmobranchs and agnatha. We were not able to find definitive ASC in either the lamprey EST or initial genome draft. However, one elasmobrach EST sequence was found that most certainly represents a true ASC ortholog from the Pacific electric ray (*Torpedo californica*: EW688743). As shown in Fig. 6, ASC from elasmobranchs through mammals possess the N-terminal PYD coupled to C-terminal CARD structure indicating this domain structure existed prior to the emergence of the gnathostomes and our inability to locate ASC or related adaptor molecules in the lamprey likely reflects the current lamprey genome assembly. Although ASC gene models (PYD-CARD) were not found in any of the invertebrate genomes, there are putative adaptors (e.g. CARD-CARD, CARD-Death, Death-Death) in both the urchin and amphioxus genomes that could interact with the vastly expanded array of NLR-like gene models in these species (Messier-Solek et al., 2010).

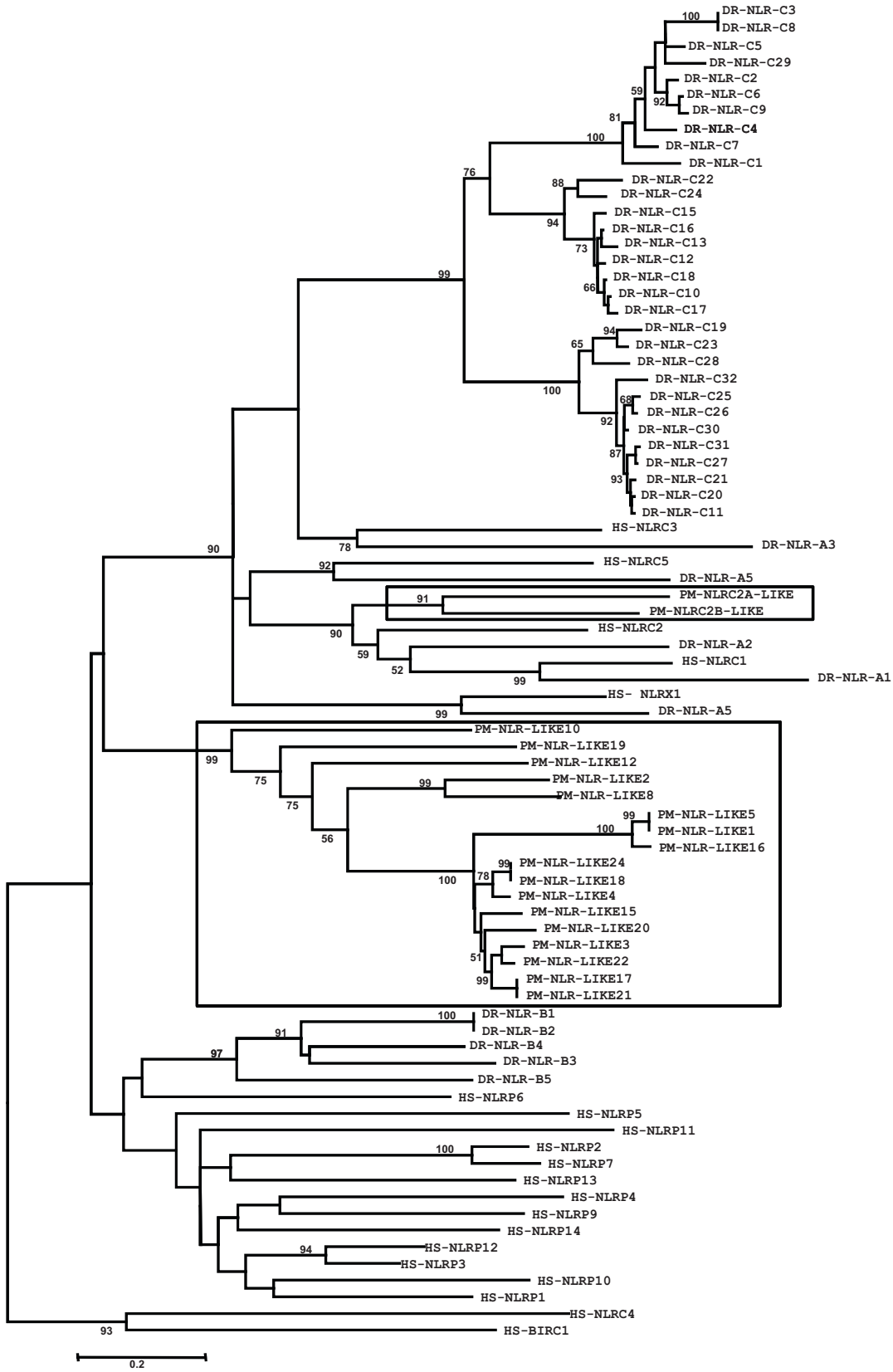

Fig. 5. Phylogenetic relationships of human, zebrafish and lamprey NLRs. ClustalW alignments of NLR NACHT domains were used to generate a Neighbor Joining tree (MEGA 4) supported by 1000 bootstrap replicants. Two lamprey models resembled NOD1/NOD2 (small box) and grouped in the NLRC clade whereas an expanded group of 17 distinct sequences formed a separate clade that is distinct from the zebrafish and human NLRP and NLR group C (labeled NLRPs in the figure) genes—large box. A total of 19 distinct NACHT domains were identified during this initial survey of the lamprey pre-build v3.0. *Abbreviations*: HS: *Homo sapiens*; DR: *Danio rerio*; PM: *Petromyzon marinus*.

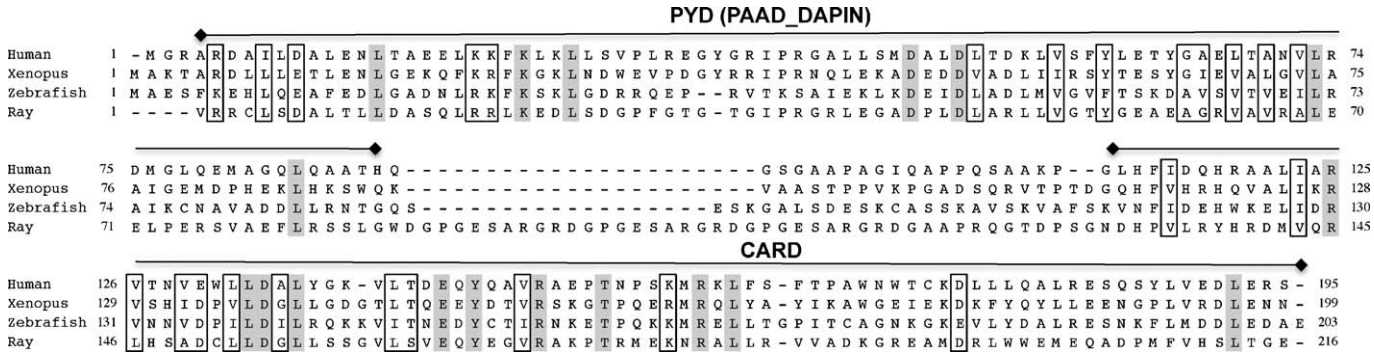

Fig. 6. Multiple alignment (ClustalW) of ASC from gnathostomes. PYD and CARD domain boundaries are based upon logos at PFAM. Sequence accession numbers were as follows: Human—ENSP00000247470, *Xenopus tropicalis*—NP_001106550, Zebrafish—ENSDARP00000059711 and Pacific electric ray (*Torpedo californica*)—EW688743. Identical amino acids are shaded, whereas those showing similarity are boxed.

A brief word on putative inflammatory caspases in fish—caspase 1 (also called intereleukin-1β converting enzyme, ICE) was first recognized in mammals nearly 20 years ago (Cerretti et al., 1992; Thornberry et al., 1992) for its ability to cleave IL-1β into an active mature form. Prototypical caspase-1 orthologs are approximately 400 amino acids in length and possess an N-terminal CARD domain followed by the CASc (caspase, IL-1β converting enzyme homologs) domain. The CARD domain is responsible for inflammasome protein–protein interactions with ASC or directly with the CARD/PYD domains in NLRs while the CASc domain is the core catalytic domain for self and substrate proteolysis. Caspase-1 orthologs have been described for zebrafish, sea bream and more recently in rainbow trout (Chang et al., 2010; Lopez-Castejon et al., 2008; Masumoto et al., 2003)—additional caspase-1 orthologs are present in the draft genomes of sticklebacks, medaka and *Tetraodon*, indicating that caspase-1 has origins predating teleosts. Interestingly, the putative caspase-1 orthologs in zebrafish (CASPYA and B) lack the N-terminal CARD that is found in other fish caspase-1 orthologs and instead possess a PYD (Masumoto et al., 2003). CASPY-A can interact with zebrafish ASC, although CASPY-associated cell death seems to align more with roles in development than in inflammation. That caspase-1 mediated cleavage of IL-1β is conserved in fish seems unlikely given that fish IL-1β typically lack the aspartic acid residue necessary for caspases-1 recognition (Bird et al., 2002; Huising et al., 2004; Wang et al., 2006). However, studies in gilthead seabream suggest that ATP-dependent IL-1β processing, mediated possibly through the purinergic receptor P2X7, within microvesicles can be blocked with known ICE inhibitors (Pelegrin et al., 2004) and that the caspase-processing function of sea bream caspase-1 protein is biologically conserved (Lopez-Castejon et al., 2008). Further studies are needed to define the exact nature of these processing events. Finally, this area of research has yet to be addressed for elasmobranchs and agnathan fish.

5. ALRs—a novel member of the PRR family

Recently a novel group of PRRs has been described. Named ALRs for AIM2-like receptors—after the founding member of this gene family is Absent in melanoma 2 (AIM2)—(Unterholzner et al., 2010) this group of cytoplasmic receptors is currently characterized only in mammals. ALRs are a small gene family that includes 4 members in humans and 6 in mice that share a characteristic hallmark IFI200 domain (also known as HIN200, Pfam domain 02760). Three of the human ALR family members; myeloid cell nuclear differentiation antigen (MNDA), pyrin and HIN domain family, member 1 (PYHIN1; also known as interferon-inducible protein X, IFIX) and AIM2 possess an N-terminal PYD coupled to the IFI200 domain whereas the fourth member, gamma-interferon-inducible protein 16 (IFI16), encodes PYD coupled to two tandem IFI200 domains.

Only AIM2 and IFI16 have been directly implicated as PRRs. Interestingly, the PYD of AIM2 can interact with ASC (Hornung et al., 2009), thus an AIM2 inflammasome can form to activate caspase 1 and produce of bioactive IL-1 family members (Fig. 3). The IFI200 domain of AIM2 can also interact with both bacterial and viral derived dsDNA (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009) leading to both of IFN production and the AIM2 inflammasome activation (Jones et al., 2010). Levels of the AIM2 inflammasome are increased during infection owing to STING (stimulator of interferon genes)-dependent mechanisms (Ishikawa et al., 2009)—thus feedback loops post-IFN production can potentiate immune response demonstrating interplay of pathogen recognition pathways. Our initial searches failed to locate HIN200 family members in teleosts although, as the HIN200 domain logo is only based upon a few available sequences from mammals, we cannot rule out their presence.

6. Concluding remarks

The ability of the innate immune system to sense and initiate specific responses to a diversity of pathogens has only recently been recognized. The basic protein subunit building blocks of the PRRs and their downstream pathways, as well as the 3 main classes of PRR (TLRs, NLRs, and RLRs) have early origins and have been conserved throughout invertebrate and vertebrate evolution, indicating their essential role in innate immunity. The lack of ALRs outside of mammals, suggest that this is a novel family of mammalian PRR. The similarity in domain architecture across PRRs implies duplication and sharing of domains both between and within the distinct PRR families. In addition, the expansion of PRRs in animals that appear to rely heavily on innate immunity, such as fish, implies that PRR diversification is a useful way to extend immune-system sensing capacity. The inherent plasticity of this system of domain trading and gene duplication and diversification may allow animals to evolve lineage-specific responses to differing pathogen pressures, while maintaining conserved pathways that result in broadly protective immune responses, such as the induction of anti-microbial genes and the processing of IL1 family cytokines.

The existence of multiple types of PRRs in different cellular compartments, some of which sense similar ligands, highlights the synergistic complexities possible in innate immune responses in a single organism. As well as being the first line of defense, the innate immune system plays an essential role in inducing and manipulating adaptive immunity. By working together or in opposition, the different outcomes induced by TLRs, RLRs, and NLRs/ALRs in the context of infection could allow for a high degree of specificity in the overall response to particular microbes. The intersection of these PRR pathways and downstream effects is an exciting area

of future research and may reveal how different animals evolve unique solutions to the pressures posed by pathogenic microbes.

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Note. While this paper was in press, Ohtani and colleagues (Ohtani et al., 2010) demonstrated that rhabdoviral infection in Japanese flounder leads to induction of LGP2 message and that subsequent transfection studies showed that full-length Japanese flounder LGP2 induces the transcription of IFN-stimulated genes, limits viral replication and most importantly—that the RD domain of fish LGP2 appears to be required for viral recognition.

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