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The versatile roles of CARDs in regulating apoptosis, inflammation, and NF-κB signaling

Wen-Pin Kao · Chao-Yu Yang · Tsung-Wei Su · Yin-Ting Wang · Yu-Chih Lo · Su-Chang Lin

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Abstract CARD subfamily is the second largest subfamily in the DD superfamily that plays important roles in regulating various signaling pathways, including but not limited to NF-kB activation signaling, apoptosis signaling and inflammatory signaling. The CARD subfamily contains 33 human CARD-containing proteins, regulating the assembly of many signaling complexes, including apoptosome, inflammsome, nodosome, the CBM complex, PIDDosome, the TRAF2 complex, and the MAVS signalosome, by homotypic CARD–CARD interactions. The mechanism of how CARDs find the right binding partner to form a specific complex remains unclear. This review uses different classification schemes to update the classification of CARD-containing proteins. Combining the classification based on domain structures, functions, associated signaling complexes, and roles would help better understand the structural and function diversity of CARD-containing proteins. This review also summarizes recent structural studies on CARDs. Especially, the CARD-containing complexes can be divided into the homodimeric, heterodimeric, oligomeric, filamentous CARD complexes and the CARD–ubiquitin complex. This review will give an overview of the versatile roles of CARDs in regulating signaling transduction, as well as the therapeutic drugs targeting CARD-containing proteins.

Keywords Apoptosis · Inflammation · NF-κB activation · CARD · Crystal structure

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Introduction

Apoptosis and inflammation are key events in immune responses. The signaling pathways leading to either apoptosis or inflammation are integrated to produce an immune response, or, when they were dysregulated, a disease [1–3]. Apoptosis was known as programmed cell death, which has a critical role in development and homeostasis in a multicellular organism [4, 5]. To against intracellular pathogens, such as viruses, apoptosis is integrated with inflammation reaction. However, in contrast to inflammation process, which depends on protein-kinase activation leading to, for example, NF-κB activation, apoptosis process relays on the activation of caspases [6, 7]. When apoptosis is out of control, it may result in serious diseases, such as infectious diseases, autoimmune diseases, neurodegeneration, and cancer [8, 9]. Some diseases are related to both apoptosis and inflammation; hence investigations in these fields have absolute biological importance [2].

Intrinsic and extrinsic apoptotic pathway

Two distinct pathways result in apoptosis activations: the intrinsic pathway and the extrinsic pathway. Both intrinsic and extrinsic pathways can activate caspases, a family of cysteine proteases that evoke a proteolytic cascade to

remove the dying cell. Various types of intracellular stress, such as growth factor withdrawal, DNA damage, heat, radiation, could induce the intrinsic (also named mitochondrial) pathway of apoptosis. B cell lymphoma 2 homology 3 (BH3)-only proteins activated by the intrinsic apoptotic stimuli then induce the activation of BAX and BAK, and mitochondrial outer membrane permeabilization (MOMP), which subsequently triggers the release of proteins from mitochondrial intermembrane space (IMS) and induces caspase activation and apoptosis [10–13]. For example, cytochrome *c* released from IMS binds Apaf-1 and induces the oligomerization of Apaf-1, which in turn forms a structure termed apoptosome that can recruit and activate pro-caspase-9 [14, 15]. Activated caspase-9 cleaves and activates executioner caspases, caspase-3 and caspase-7, in order to execute apoptosis [16]. In addition, mitochondrial release of Smac and OMI neutralizes the caspase inhibitory function of inhibitor of apoptosis (IAP) [10, 17].

Stimulation through the death receptors (DRs) could induce the extrinsic apoptotic pathway. Activation of DRs by the binding of its ligands results in the oligomerization of DRs and the subsequent recruitment of adaptor proteins to form a signaling complex that could recruit procaspase-8/-10 [18]. There are two types of DR signaling complexes. One is composed of the receptor Fas, DR4, or DR5, and the adaptor protein Fas-Associated protein with Death Domain (FADD). The other is composed of receptor TNFR1, DR3, DR6, or EDAR, and the adaptor protein Tumor necrosis factor Receptor type 1-Associated Death Domain protein (TRADD). The formation of DR signaling complexes could trigger the dimerization and activation of procaspase-8/-10 for the activation of caspase-3 and caspase-7 to execute apoptosis [19]. Noteworthily, stimulation through DRs can also induce, however, the activation of NF-kB signaling pathway [20].

Death domain superfamily and homotypic interactions

The assembly of signaling complexes is a common key event of both caspase activation and NF-κB activation. The death domain (DD) superfamily plays a pivotal role in the formation of signaling complex. The DD superfamily can be classified into four subfamilies: death domain (DD), death effector domain (DED), caspase recruitment domain (CARD) and pyrin domain (PYD) [21]. Sequence-based phylogenetic analysis suggests that the father of the death domain superfamily gave birth to a CARD and a DD–DED–PYD ancestor. The latter then evolved into a DD and a DED–PYD ancestor. The DED–PYD ancestor in turn evolved into a DED and a PYD ancestor [22]. Although all subfamily members are derived from the same ancestor, interestingly, homotypic interactions are present only between the proteins in the same subfamily, which are located throughout different pathways of caspase activation, apoptosis signaling, and NF-kB activation. Structural studies have showed how an Apaf-1 molecule interacts with a caspase-9 molecule via homotypic CARD–CARD interaction [23], and also how a Pelle molecule interacts with a Tube molecule via homotypic DD interaction [24]. Amazingly, recent structural studies of the signaling complexes of PIDD-RAIDD [25], MyD88-IRAK4-IRAK2 [26], Fas-FADD [27, 28], and RIG-I-MAVS [29] revealed that DD superfamily members actually use more complicated homotypic interactions to form a signaling complex. These studies suggest that the surface and charge complementarity are important for the specificity of homotypic interactions, which allow different members in the same subfamily to form different signaling complexes, respectively.

The CARD subfamily

The CARD subfamily proteins were first described as a motif that interacts with caspases and the adaptor molecules of caspase. Homotypic CARD–CARD interaction between Apaf-1 and caspase-9 plays a critical role in caspase-9 activation and apoptosis [30]. However, recent studies showed that many CARD-containing proteins mediate the assembly of proteins in apoptosis, NF-κB signaling, and inflammation [31, 32]. There are 33 human CARD-containing proteins identified and participating a broad spectrum of signaling pathways [2, 33]. It is worthy to put more effort into unraveling the mechanisms of CARD-containing protein-mediated signaling by understanding the CARD structures. In this review, we will describe the classification of CARD-containing proteins and a progress of structural study of CARD-containing proteins so far.

CARD classification based on structural features

Previous reviews tried to classify CARD-containing proteins based on either structures or functions. Based on overall domain structures of proteins, CARD-containing proteins can be divided into four subgroups [34]. Table 1 shows the updated, six subgroups, classification of CARD-containing proteins based on their overall domain structures. Most CARD-containing proteins in the first three subgroups have multiple domains (Fig. 1c). The first subgroup is NBD-CARDs. In addition to a CARD module, the protein in this subgroup has a nucleotide-binding domain (NBD or NACHT) which likely functions in oligomerization. It also

Table 1 Classification of CARD-containing proteins by domain structures, functions, associated signaling complexes, or their roles

Sub-groups	Proteins	Functions	Signaling complexes	Main roles
NBD-CARDs	Apaf-1	Caspase-9 activation	Apoptosome	Receptor
	NLRC4/IPAF/CARD12	Caspase-1 activation	Inflammasome	Receptor
	NLRP1/NALP1/CARD7	Caspase-1 activation	Inflammasome	Receptor
	NOD1/CARD4	NF-κB activation	Nodosome	Receptor
	NOD2/CARD15	NF-κB activation	Nodosome	Receptor
Coiled-coil CARDs	CARD9	NF-κB activation	CBM complex	Regulator
	CARD11/CARMA1	NF-κB activation	CBM complex	Regulator
	CARD14/CARMA2	NF-κB activation	CBM complex	Regulator
	CARD10/CARMA3	NF-κB activation	CBM complex	Regulator
	DLG5	Unsolved	Unsolved	Unsolved
Multipartite-CARDs	cIAP1	NF-κB activation, Caspase inhibition	TRAF2 complex	Regulator
	cIAP2	NF-κB activation, Caspase inhibition	TRAF2 complex	Regulator
	MDA5	NF-κB activation	MAVS signalosome	Receptor
	RIG-I	NF-κB activation	MAVS signalosome	Receptor
Bipartite-CARDs	ASC/PYCARD	Caspase-1 activation	Inflammasome	Adaptor
	CARD8/CARDINAL	Inhibit NF-κB activation, Inhibit Caspase-9 activation	Inflammasome	Inhibitor
	Caspase-1, -4, -5, -12	Inflammation	Inflammasome	Initiator/Effector
	Caspase-2	Apoptosis	PIDDosome	Initiator/Effector
	Caspase-9	Caspase activation	Apoptosome	Initiator/Effector
	RAIDD/CRADD	Caspase-2 activation	PIDDosome	Adaptor
	RIPK2/RICK/RIP2	NF-κB activation	Nodosome	Effector
Shorthair-CARD	Bcl10	NF-κB activation	CBM complex	Adaptor
	BinCARD/C9orf89	Inhibit NF- κB activation	CBM complex	Inhibitor
	CARD16/COP	Inhibit Caspase-1 activation	Inflammasome	Inhibitor
	CARD17/INCA	Inhibit Caspase-1 activation	Inflammasome	Inhibitor
	CARD18/ICEBERG	Inhibit Caspase-1 activation	Inflammasome	Inhibitor
	NOL3/ARC	Inhibit Caspase-2, -8 activation	Unsolved	Inhibitor
Longhair-CARD	CARD6	NF-κB activation	Unsolved	Regulator
	MAVS	NF-κB activation	MAVS signalosome	Adaptor
	QRICH1	Unsolved	Unsolved	Unsolved

contains a leucine-rich repeat domain or WD-40 domain, which acts as a sensory domain to regulate its oligomerization [35]. The second group is coiled-coil CARDs. Apart from the similar structural features of NBD-CARDs, these proteins have a coiled-coil motif to substitute for NBD and to function in oligomerization. Instead of having a sensory domain, they have a C-terminal MAGUK domain responsible for their membrane localization [36, 37]. These two groups of proteins are more likely to act as scaffold proteins in the formation of activation complexes.

The CARD-containing proteins of multiple domains, if not included in the first and second group, belong to the third group as a multipartite-CARD (Table 1; Fig. 1c). Currently, there are four members in this group, including cIAP1, cIAP2, MDA5, and RIG-I. Both cIAP1 and cIAP2 have three BIR domains that mediate protein–protein interaction, a UBA domain responsible for ubiquitin-binding, a CARD required for cIAP autoregulation [38], and a RING domain with ubiquitin ligase (E3) activity [39]; RIG-I and MDA5 both have two CARD modules responsible for downstream MAVS recruitment, protein–protein interaction, and ubiquitin-binding. They also have a helicase domain and a CTD for sensing viral RNA [40, 41]. Briefly, the proteins in the first three subgroups have multiple domains and are multi-functional.

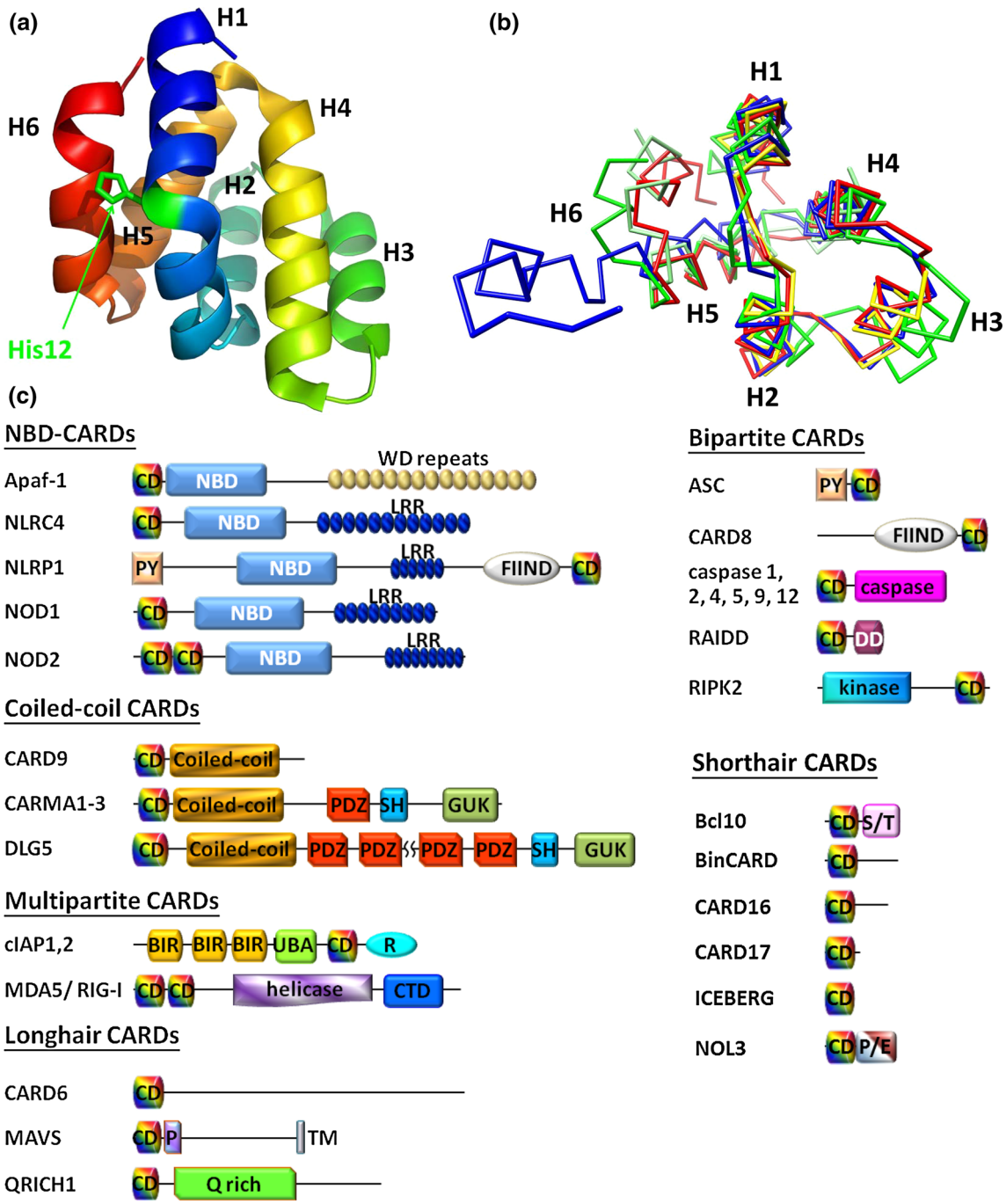

Fig. 1 The structure of CARD and the domain structures of CARD-containing proteins. **a** Apaf-1 CARD contains a six-helices bundle (H1–H6) with a kink in H1 (His12) (PDB: 1CY5). **b** Superimposition of the CARD structures, including *Red*: Apaf-1 (PDB: 1CY5), *Green*: cIAP1 (PDB: 2L9 M), *Blue*: NOD1 (PDB: 2NSN), *Yellow*: CARMA1 (PDB: 4JUP) and *Purple*: ICEBERG (1DGN). This is the *bottom view* of (**a**). **c** The domain structures of CARD-containing proteins. *CD* CARD caspase recruitment domain; *NBD* nucleotide-binding domain NACHT; *WD repeat* beta-transduction repeat, often terminate in a W-D dipeptide; *LRR* leucine-rich repeat domain; *PY* PYD pyrin domain; *FIIND* function to find domain; *PDZ* PSD95, DLG1, and ZO-1; *SH* SH3 domain, Src homology 3 domain; *GUK* guanylate kinase domain; *BIR* baculovirus IAP repeat domain; *UBA* ubiquitin-associated domain; *R* RING, Really Interesting New Gene domain; *CTD* C-terminal domain; *DD* death domain; *S/T* Ser/Thr rich motif; *P/E* Pro/Glu rich motif; *P* Proline rich motif; *TM* transmembrane domain; *Q rich* Glutamine rich motif

The fourth group of proteins is usually enrolled by NBD- or coiled-coil-CARDs and becomes activated in the process, and what is more, they can recruit effector molecules to the complex. Hence, these proteins were named bipartite-CARDs, which contain a CARD and one other domain. For example, ASC has a PYD that can

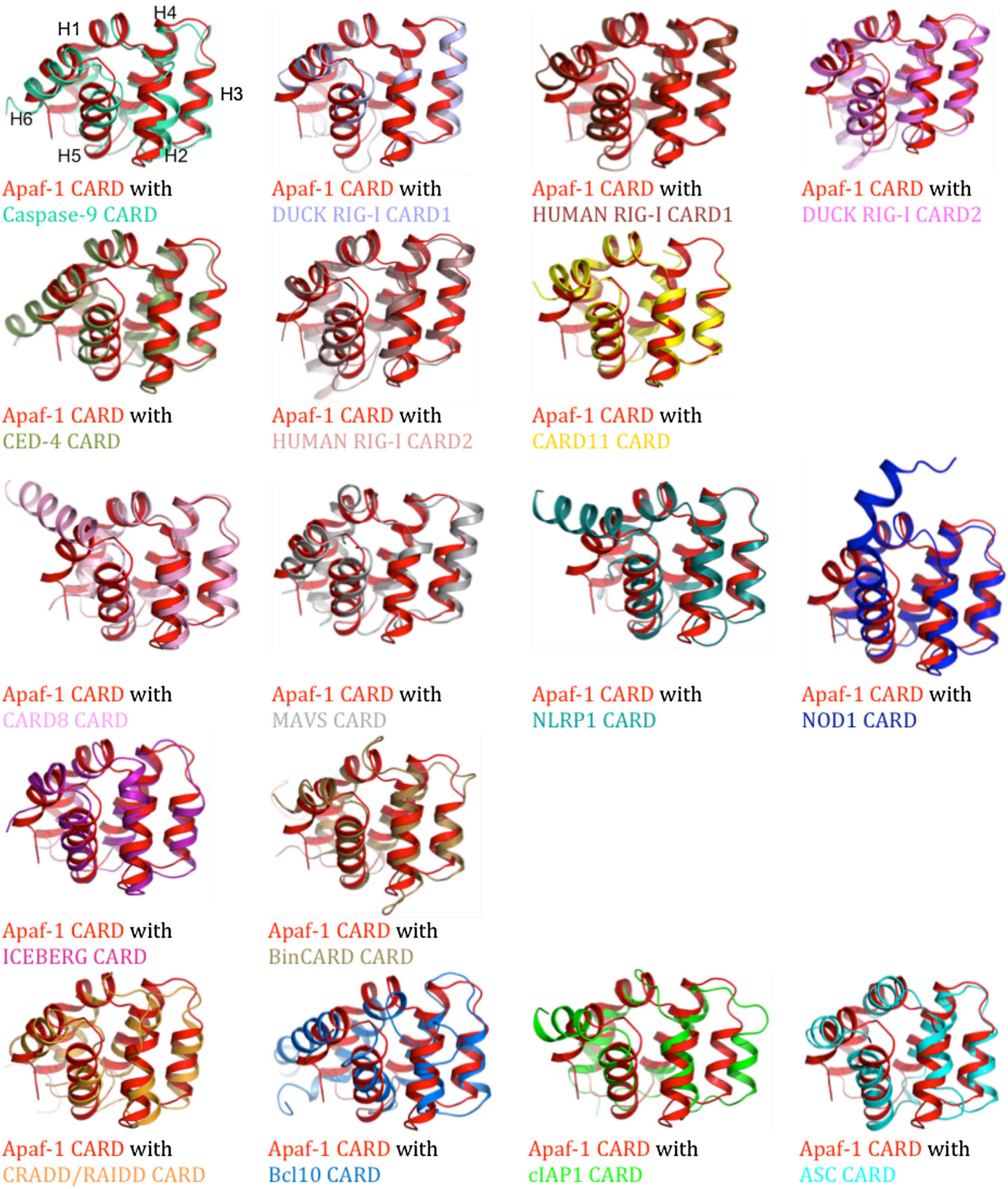

Fig. 2 Structure comparison of CARDs. The CARDs of ASC, Bcl10, BinCARD, caspase-9, CARD8, CARD11, CED-4, cIAP1, duck RIG-I, human RIG-I, ICEBERG, MAVS, NOD1, NLRP1, and RAIDD, are superimposed with Apaf-1, respectively. The structures are listed, from *left* to *right*, in descending order of structure similarity by Z score, with the name under the structure. Six helices are indicated as H1–H6. Structural superimposition was done by pairwise DALI and represented by Pymol. The results of pairwise DALI are summarized in Table 2

interact with the PYD of the activated NLRs, like NLRP3. ASC then recruits procaspase-1 through CARD–CARD interaction for the production of mature IL-1beta by activated caspase-1 [42]; Caspase-9 has a CARD that can interact with Apaf-1 CARD in apoptosome for caspase autoactivation. Active caspase-9 then cleaves effector caspases, such as caspase-3 and caspase-7 [43]; RAIDD contains a DD that can interact with the DD of the activated receptor PIDD. RAIDD then recruits procaspase-2 through CARD–CARD interaction for executing apoptosis [44]; RIPK2 has a CARD for the recruitment by activated NOD1 or NOD2 receptor. Subsequently, the activated and ubiquitinated RIPK2 can activate downstream kinases for NF-kB activation [45]. In summary, the proteins of the fourth group are usually bi-functional.

The fifth group is shorthair-CARD proteins, which do not hold any additional domains, but may have additional sequence or motif. For example, Bcl10 contains a CARD and a C-terminal S/T rich motif. Upon activation, CARMA1 can form an oligomer through the coiled-coil motif and then sequentially recruit Bcl10 molecules by CARD–CARD interaction and also MALT1 [46]. BinCARD, however, can inhibit Bcl10-mediated NF-kB activation through direct CARD–CARD interaction with Bcl-10 [47]. CARD16, 17, and18 are truly CARD-only proteins, which share a high degree of identity to the prodomain of caspase-1. All of them can interact with procaspase-1 to block the release of mature IL-1 beta [48–51]. NOL3/ARC has a CARD and a C-terminal P/E rich motif. It can interact with caspase-2 and caspase-8 to down-regulate their activity [52]. Surprisingly, NOL3/ARC could disrupt the extrinsic pathway by nonhomotypic interactions between NOL3/ARC CARD and the DDs of the Fas and FADD [53], which is the only known exception to the homotypic interactions in the DD superfamily.

The last group is longhair-CARDs. The members in this group have a CARD followed by a long sequence without any obvious functional domain. CARD6 has 1,033 amino acids, which can associate with microtubule and RIPK2. The CARD of CARD6, however, negatively controls their association [54]. MAVS, 540 amino-acid long, has a CARD at the N-terminus and a transmembrane region at the C-terminus. MAVS CARD can interact with the CARD of viral RNA sensor RIG-I or MDA5, which in turn transduces the signal to activate NF-kB through a unknown mechanism [55]. QRICH1 has 776 amino acids, however, with unknown functions.

CARD classification based on functions

Hong and Jung had tried to classify CARD-containing proteins according to their functions. CARD was thought as a bi-functional switch of caspase regulation and NF-kB activation [31]. Unlike the original identification, CARDs are not only interacting with caspase but also engaging in mediating the assembly of the signaling complexes in apoptosis and NF-κB activation. Table 1 shows the updated functions of CARD-containing proteins. CARDs function in at least three different pathways: caspase

Table 2 Structural comparison statistics of the CARD structures. Structural comparison between Apaf-1 CARD and other CARDs was done by pairwise DALI. The superimposed structures are displayed in Fig. 2

Protein and accession number	Method	r.m.s.d	Seq. Identity (%)	Z-score	Reference
Apaf-1 (1CY5)	X-ray diffraction	–	–	–	[71]
Caspase-9 (3YGS)	X-ray diffraction	1.5	20	15.2	[23]
Duck RIG-I CARD1 (4A2Q)	X-ray diffraction	1.4	22	14.9	[80]
RIG-I CARD1 (4NQK)	X-ray diffraction	1.4	21	14.3	[79]
Duck RIG-I CARD2 (4A2Q)	X-ray diffraction	1.4	15	13.0	[80]
CED-4 (2A5Y)	X-ray diffraction	1.8	15	12.9	[75]
RIG-I CARD2 (4NQK)	X-ray diffraction	1.6	16	12.9	[79]
CARD11 (CARMA1)(4JUP)	X-ray diffraction	1.5	22	12.8	[74]
CARD8 (4IKM)	X-ray diffraction	2.3	19	12.5	[73]
MAVS (IPS-1)(2VGQ)	X-ray diffraction	1.8	14	12.2	[76]
NLRP1 (4IFP)	X-ray diffraction	2.3	20	11.7	[77]
NOD1 (2NSN)	X-ray diffraction	1.5	19	11.6	[78]
ICEBERG (1DGN)	NMR	2.0	24	10.9	[70]
BinCARD (C9orf89) (4DWN)	X-ray diffraction	2.3	22	10.8	[46]
CRADD/RAIDD (3CRD)	NMR	2.4	14	9.3	[69]
Bcl10 (2MB9)	NMR	2.6	13	8.1	[68]
cIAP1 (2L9 M)	NMR	2.6	21	7.3	[38]
ASC (2KN6)	NMR	2.9	23	6.8	[67]

activation in the process of apoptosis, caspase activation in the process of inflammation, and NF-κB activation in immune responses. They also function in the inhibitory regulation in the processes mentioned above. Apparently, CARD-containing proteins could be like a multifunction switch that could regulate caspase activation in apoptosis and inflammation, and also regulate NF-kB activation.

As shown in the Table 1, most CARD-containing proteins with multiple domain structures and longhair-CARDs function in the NF-κB activation pathway. Most NBD-CARDs and shorthair-CARDs function in caspase activation and inhibitory regulation, respectively. The functions of bipartite-CARDs are, however, quite diverse. It seems that most CARD-containing proteins in the same pathway would have different domain structures. Combining with the classification based on structural domains suggests that more classification schemes may help better understand the versatile roles of CARD-containing proteins.

CARD classification based on signaling complexes and roles

Assembly of signaling complexes is a key event in intracellular signaling. DD superfamily members could form different signaling complexes in different signaling pathways. Most CARD-containing proteins are associated with different size of signaling complexes through CARD–CARD interactions. Especially, in a signaling complex, each CARD-containing protein may play different roles. To find out every CARD-containing protein in a signaling complex and its role may help better understand the versatile roles of CARD-containing proteins. Here we try to classify CARD-containing proteins based on the associated signaling complexes and their roles (Table 1; Figs. 3, 4). There are at least seven CARD-containing signaling complexes, include apoptosome, inflammasome, nodosome, the CBM complex, the TRAF2 complex, the MAVS signalosome, and PIDDosome.

Apoptosome comprises seven Apaf-1 and seven cytochrome c molecules. Apaf-1 may act as a receptor for sensing apoptotic signals, e.g. cytochrome c, from the intrinsic pathway. When Apaf-1 binds to cytochrome c, Apaf-1 is activated and then together form a wheel-like structure in order to activate effector caspases [56]. There are two CARD-containing proteins, Apaf-1 and caspase-9, in apoptosome signaling pathway. Apaf-1 can recruit procaspase-9 by CARD–CARD interactions in order to activate procaspase-9. Activated caspase-9 may act as an initiator or effector to activate downstream procaspase-3 and -7 [14].

A large multimolecular complex, inflammasome, is a key component of innate immunity, which regulates the activation of procaspase-1 for inflammatory processes. The activation of caspase-1 mediates the maturation of proinflammatory cytokines, interleukin-1β (IL-1β) and IL-18

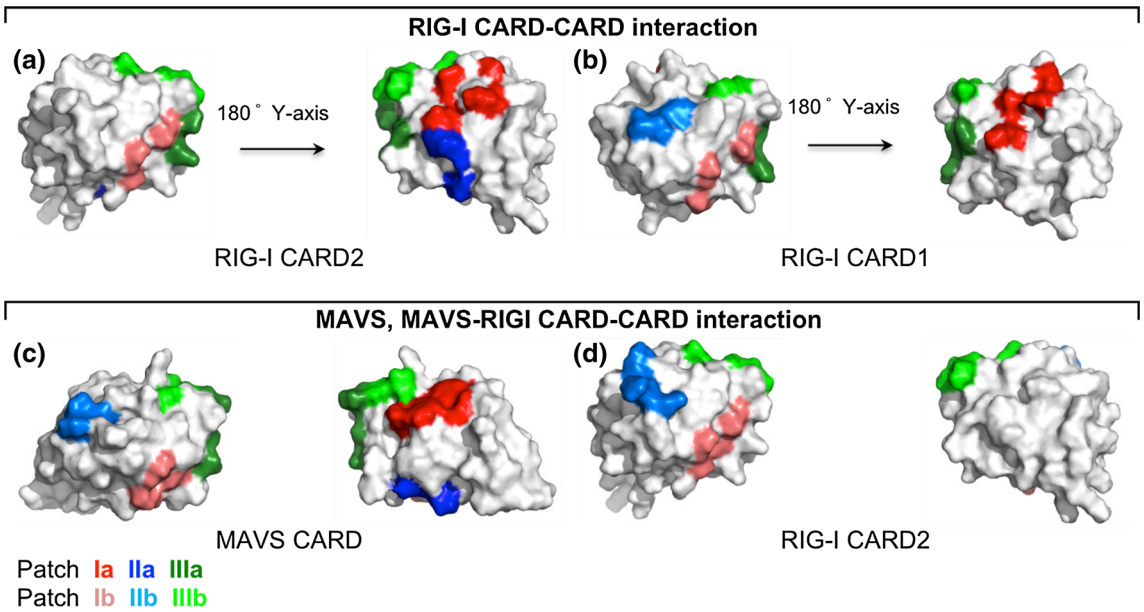

Fig. 3 Surface presentation of the type I, II, III interfaces. The type I, II, and III interfaces were derived from the structures of RIG-I-MAVS signalosome (PDB ID: 4P4H, and 3J6 J). **a**, **b** The interfaces identified between RIG-I CARDs and mapped on CARD1 (**a**) and CARD2 (**b**), respectively. **c**, **d** The interfaces identified between MAVS CARDs and also between MAVS CARDs and RIG-I CARDs. The interfaces are mapped on MAVS (**c**) and RIGI (**d**), respectively, and are colored using the color indicated *below*. The orientation of models is the same as in Fig. 2. The surface on the right is generated by rotating the model on the left by 180° along the Y-axis

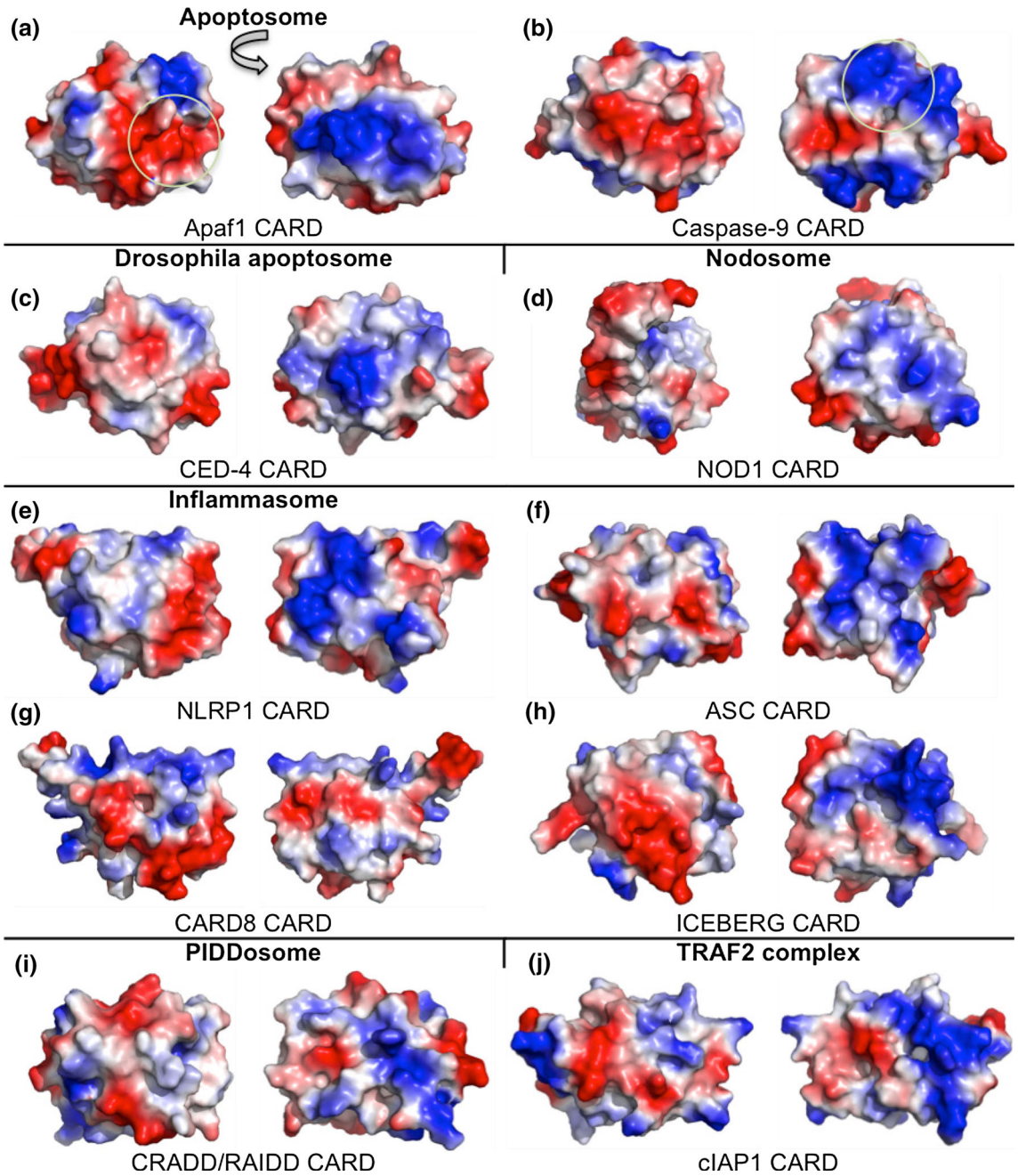

Fig. 4 Electrostatic surface representation of CARDs. Each CARD on the *left* is shown in the same orientation as in Fig. 2. The CARD-associated signaling complexes shown here include human apoptosome (**a** Apaf-1 and **b** caspase-9), Drosophila apoptosome (**c** CED-4), nodosome (**d** NOD1), inflammasome (**e** NLRP1, **f** ASC, **g** CARD8, and **h** ICEBERG), PIDDosome (**i** CRADD/RAIDD), and the TRAF2 complex (**j** cIAP1). The surface on the *right* is represented by rotating the model on the *left* by 180° along the Y-axis. The *circles* label the type I interface between Apaf-1 and caspase-9

[57]. In addition, caspase-1 is also involved in the cell death, called pyroptosis [58]. Two protein families are involving the formation of inflammasome: NLR and PYHIN family. Members of NLRs family, NLRP1, NLRP3, NLRC4, NLRP6 and NLRP12 have been observed in the inflammasome assembly. NLR molecules have a central NBD (or NACHT domain), a leucine-rich repeat domain, and a CARD or PYD (Fig. 1c). The activation of signaling induces the oligomerization of NLRs through their NBDs, which in turn can recruit procaspase-1. Two CARD-containing proteins, NLRC4 and NLRP1, can directly interact with procaspase-1. However, most NLRs lack CARD. Instead, they use PYD to recruit ASC involved in inflammasome formation. ASC functions to be an adaptor protein

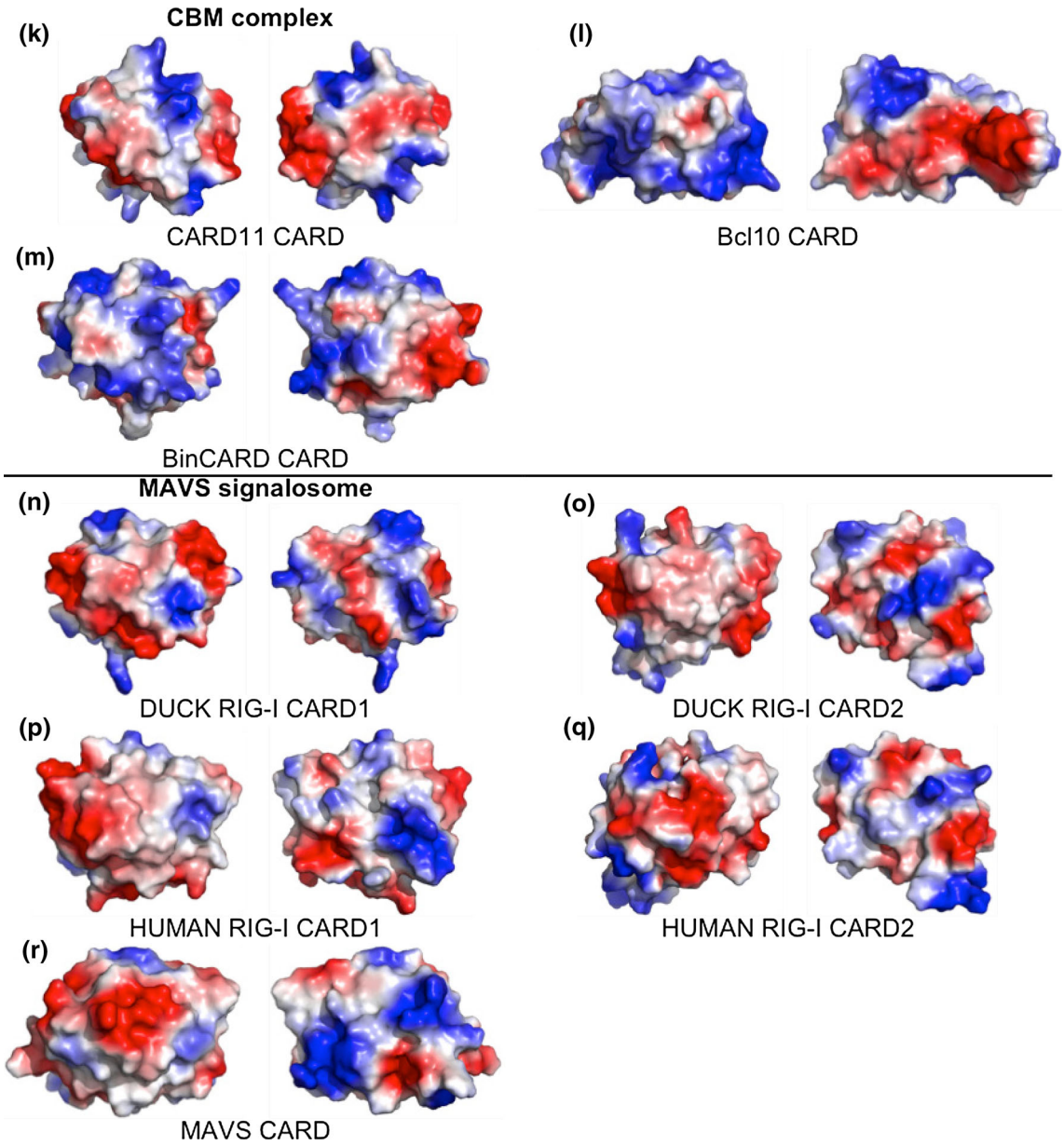

Fig. 5 Electrostatic surface representation of CARDs *(contined)*. Surface representation of CARDs is shown in the same way as in Fig. 4. The signaling complexes shown here include the CBM complex (**k** CARD11, **l** Bcl10, and **m** BinCARD) and the MAVS signalosome (**n**, **o** duck RIG-I, **p**, **q** human RIG-I, and **r** MAVS)

bridging the interaction between NLRs, which lack CARD, and procaspase-1 through CARD–CARD interactions [57].

In the nodosome complex, NOD1, NOD2, and RIPK are CARD-containing proteins. NOD1 and NOD2 are both receptors that could sense different bacterial peptidoglycan. Both NOD1 and NOD2 can form a signaling complex called nodosome by self-oligomerization, which supposedly looks similar to the wheel-like structure of apoptosome. Upon activation and subsequent conformational change, NBDs of NOD receptors assemble and then the exposed CARDs recruit effector RIPK2 by CARD–CARD interaction. The recruited RIPK2 can further activate downstream kinases for NF-kB activation [59].

A family of CARMA, a kind of scaffold protein, plays a pivotal role in the activation and recruitment of IKK for NF-κB activation. A member of CARMA family, CARMA1, interacts with two downstream signaling molecules, Bcl10 and MALT1, to form a complex termed CBM complex. CARMA1 and Bcl10 can interact with each other through homotypic CARD–CARD interaction; in contrast, owing to lack of CARD, MALT1 binds to Ser/Thr rich motif of Bcl10 [60]. Bcl10 CARD has been suggested to

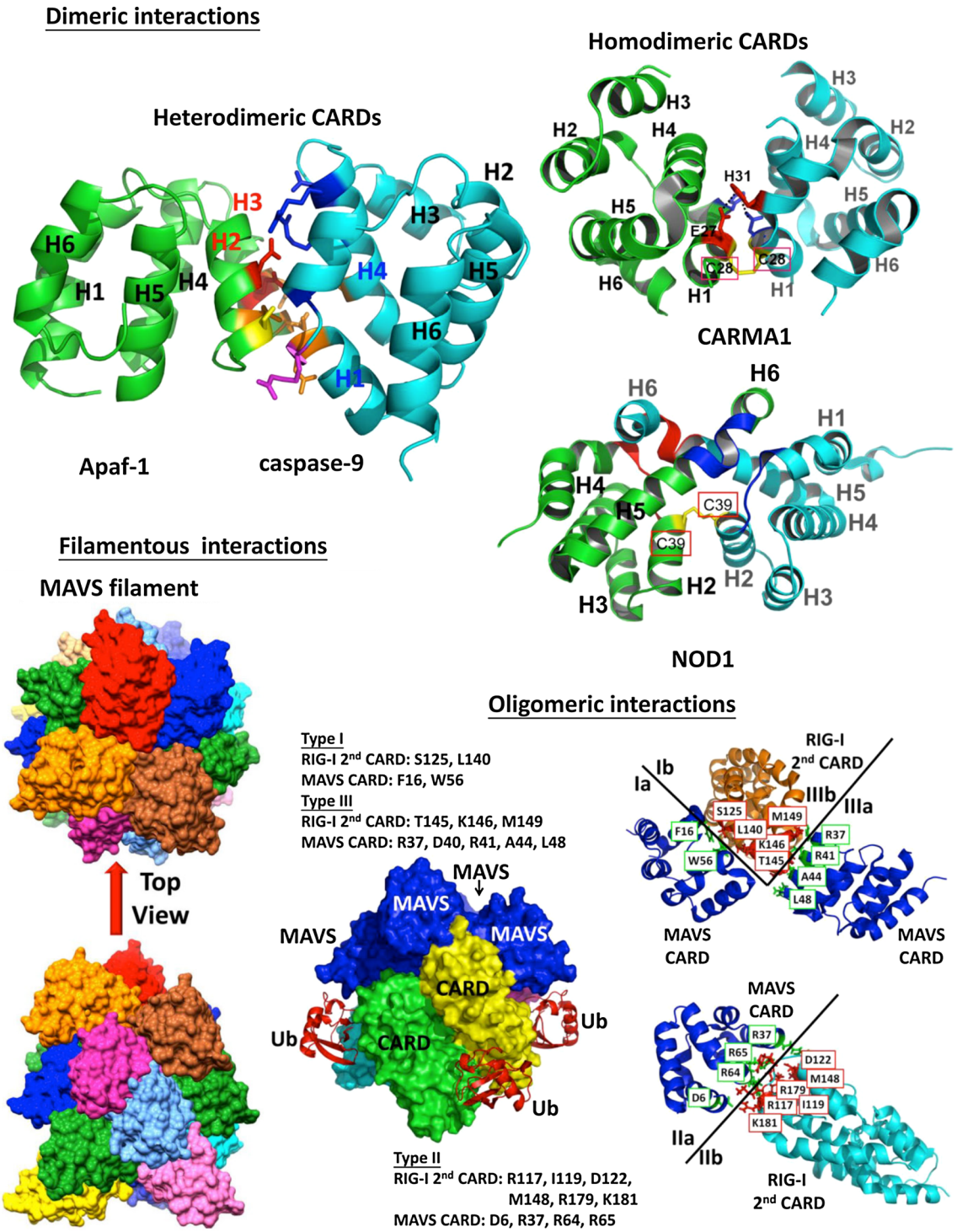

Fig. 6 Crystal structure of the CARD complexes. Apaf-1 interacts with caspase-9 to form a heterodimeric structure through the type I interface (PDB: 3YGS). In the homodimeric complexes, CARMA1 (PDB: 4JUP) and NOD1 (PDB: 2N7Z) CARDs assemble together by a disulfide bond and also electrostatic conjugation. RIG-I tandem CARD tetramer acts as a platform for MAVS assembly (PDB: 4P4H). MAVS CARDs form a single-stranded left-handed helical filament through the type I, II and III surfaces (PDB: 3J6 J). In addition, NOD1 can also form a hexameric complex through disulfide bond interaction and domain swapping (structure not shown)

function in TCR-induced actin oligomerization, and also Bcl10 oligomers act as a scaffold protein for IKK and JNK pathways. The multiple immunoreceptor tyrosine-based activation motif (ITAM) induced NF-κB activation can also be regulated by the CBM complex. Further, the Bcl10-MALT1 complex induces the release of IL-6 and TNFα.

CARD9, another CARD-containing protein similar to CARMA family proteins, can also form a complex with Bcl10 and MALT1 to activate IKK pathway [61].

The TRAF2 complex here is restricted to the IAP-containing TRAF2 complex. cIAP1/2 have an essential role in both canonical and noncanonical NF-κB signaling pathway by acting as E3 ligase. An E3 complex, including cIAP1/2 and TRAF2, mediates the activation of NIK in noncanonical NF-κB signaling pathway. NIK is then phosphorylating IKKα and activating NF-κB. TRAF2 and cIAP1/2 are also important in regulating the activation of canonical NF-κB pathway induced by TNFα, which depends on the interaction between TRAF2 and cIAP1/2. cIAP1/2 contain three N-terminal BIR domains, a UBA domain, a CARD, and a RING domain. cIAP1/2 can directly interact with TRAF2 through BIR domain to form a complex [62]. The CARD of cIAP1, however, mediates its autoinhibition [38, 63].

MAVS signalosome is an important signaling complex responsible for antiviral responses in innate immunity. Two key receptors, RIG-I and MDA5, can sense double-stranded viral RNA. When the helicase domain of RIG-I or MDA5 detects viral RNA in the cytoplasm, their CARD would be exposed in order to interact with N-terminal MAVS CARD thought CARD–CARD interaction. The interaction would result in the oligomerization of MAVS on the mitochondria, which would lead to NF-kB activation and type I interferon production [64].

PIDDosome consists of several p53-inducible death domain-containing protein (PIDD), RAIDD, and procaspase-2 molecules, which form a ring-like signaling complex. PIDDosome is a platform to activate procaspase-2 through the CARD–CARD interaction between RAIDD and procaspase-2. Assembly of PIDDosome seems to response for DNA damage. However, recent findings indicate multiple functions of different PIDDosome. For example, PIDD can auto-process to produce several isoforms with different C-terminal fragments. As PIDD-C is generated, the repair system can be triggered by the formation of NEMO PIDDosome for NF-kB activation, which does not contain RAIDD. For clarify, the PIDDosome that can activate caspase-2 to induce apoptosis signaling is also called caspase-2 PIDDosome or RAIDD PIDDosome [65]. Interestingly, caspase-2 has also been found in CD95-DISC signaling in response to DNA damage, possibly through the CARD–CARD interaction between procaspse-2 and procaspase-8 [66].

Combining all classification schemes based on structure, function, associated signaling complex, and role provides more information to better understand the versatile roles of CARD-containing proteins (Table 2). There are about 30 CARD-containing proteins identified in seven different signaling complexes. In each signaling complex, each protein has its unique role in correctly transducing either apoptotic, inflammatory, or NF-kB activating signal, or inhibiting the signal transduction. Obviously, the unique role of each CARD-containing protein in a signaling complex largely depends on its unique CARD to achieve the specificity and specialty. Structural studies on either CARD alone or CARD-containing complex would provide the information showing how CARD achieves the specificity.

The CARD structure

CARD is a small protein–protein interaction module observed in proteins involved in the inflammation and apoptosis processes. Up to now, 11 crystal structures and 5 NMR structures have been discovered (Table 1). NMR-structures are available for the CARD of ASC [67], Bcl10 [68], cIAP1 [38], CRADD/RAIDD [69] and ICEBERG [70]. Crystal structures are available for Apaf-1 [71], BinCARD [72], CARD8 [73], CARD11 [74], CED-4 [75], caspase-9 [23], MAVS [76], NLRP1 [77], NOD1 [78], and RIG-I [79, 80]. The amino acid sequence identity between CARDs is 13 and 24 % (Table 2). CARDs adopt the conserved six-helical bundle of the DD superfamily with a central hydrophobic core [34]. A unique structural feature of CARD is it inclines to form a kink in helix H1 (Fig. 1a). For example, H1 of Apaf-1 is severed kinked at His12, which induces an unusual bent structure to form separated H1a and H1b helices [71]. When superimposed, some helices have a certain extent different orientations and lengths between different CARDs (Figs. 1b, 2) [23].

Three conserved interaction surfaces in homotypic DD interactions

Members of the DD superfamily form oligomers using three types of conserved interaction surfaces. The type I interaction is through the positively charged Ia surface of one death domain (H1 and H4 helices) and the negatively charged Ib surface of another death domain (H2 and H3 helices). Regarding the type II interaction, H4 and the loop between H4 and H5 of one death domain form the type IIa surface, which can interact with the type IIb surface, located on a groove formed by H1 and H2 on one side with H6 and its preceding loop on the other side. Finally the interaction between H3 of one death domain (type IIIa surface) and a groove formed by the H1-H2 and H3-H4 loops on another death domain (type IIIb surface) forms the type III interaction surface. This interface contains hydrophobic, charged and polar interactions [81].

The recently solved structure of the CARD complex of MAVS signalosome has shown that the locations of the type I, II, and III surfaces on CARD (Fig. 3) are similar to those on DD. It's been shown that surface and charge

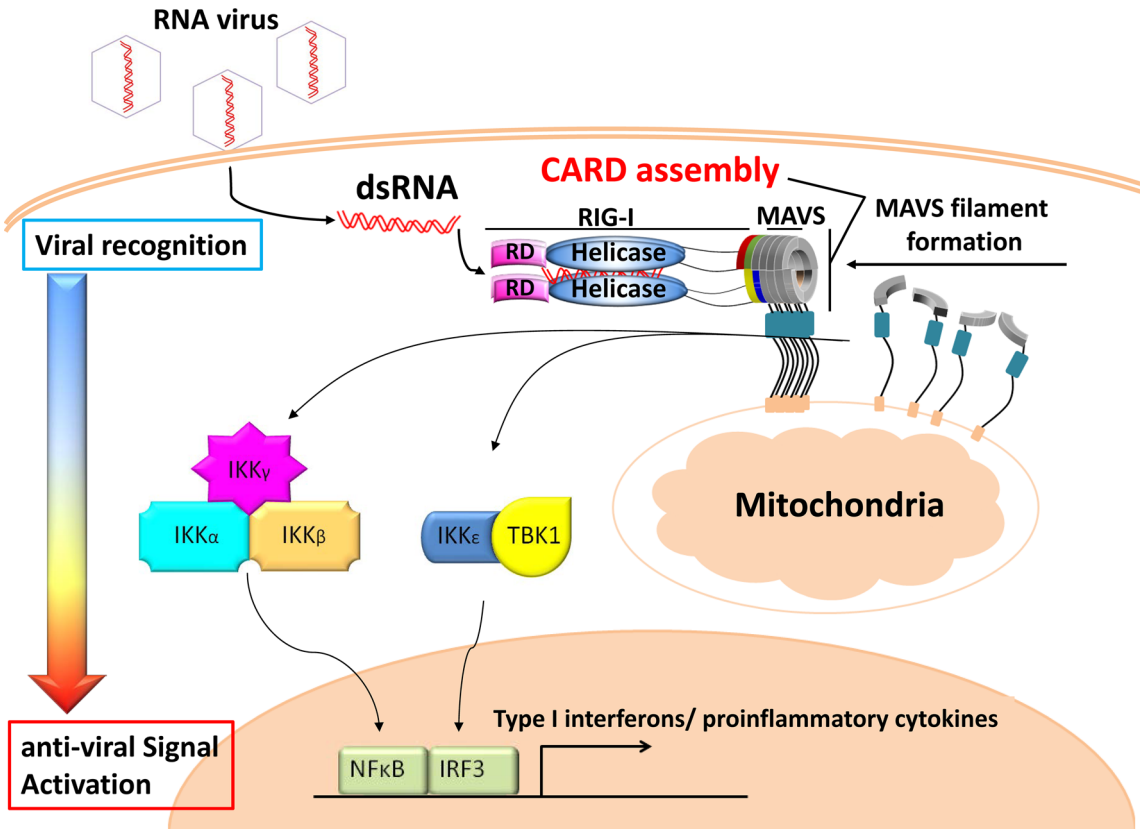

Fig. 7 The model of RIG-I mediated antiviral responses. When the C-terminal DEAD/DEAH and helicase domains of RIG-I recognize dsRNA of virus, RIG-I tandem CARD molecules form a tandem CARDs tetramer. The tandem CARDs tetramer is termed a 'lock washer', with two ends adjacent to half the thickness of the ring. This 'lock-washer' is served for MAVS-CARD filament formation and triggers the downstream signaling, such as type I interferon activation and the release of proinflammatory cytokines (Adopted from [29])

complementarity is a key for the binding specificity in Myddosome formation [26]. The polarized surface of CARDs with acidic, basic, and hydrophobic patches as well as the shape of the surface would thus be an important feature for specific CARD–CARD interaction (Figs. 4, 5). Indeed, the type I interface for Apaf-1 and caspase-9 interaction shows a charge complementary (Fig. 4). However, how the rest of CARDs form a specific complex, and how do they form a CARD-specific complex remain unclear. Currently CARD-mediated interactions observed in the structural studies could be divided into five different types of interactions, including heterodimeric, homodimeric, oligomeric, and filamentous CARD–CARD interactions, and CARD–Ub interactions, which show that CARDs play a more versatile role than other DD superfamily members.

Heterodimeric CARD–CARD interaction

Heterodimeric CARD–CARD interaction of Apaf-1 and caspase-9 is the first homotypic CARD–CARD interaction revealed by X-ray crystallography [23]. The crystal structure of the Apaf-1-caspase-9 complex gives an illustration of CARD–CARD interaction (Figs. 4, 6), in which positively charged H1 and H4 helices (R13, R52, and R56) of Caspase-9 CARD form a concave surface and recognize a negatively convex surface of Apaf-1 formed by H2 and H3 helices (D27 and E40). A hydrogen bond network between two residues of Apaf-1, D27 and E40, and four residues of caspase-9, R13, R52, D53, and R56, contributes to the interaction. D27 of Apaf-1 and R56 of caspase are located in the center of this network. I30 and I37 of Apaf-1 and I60 of caspase-9 also contribute to the interaction through van der Waals interactions. The interaction surface between Apaf-1 and caspase-9 belongs to the conserved type I interface in homotypic DD interactions.

Homodimeric CARD–CARD interaction

The disulfide bond mediated homodimeric structures of CARDs show a unique CARD–CARD interaction only present in CARD subfamily. NOD1 and CARMA1 CARDs

both form a disulfide bond-linked CARD dimer, as reveal by their crystal structures [74, 78, 82]. CARMA1 CARDs form a symmetry homodimeric CARD structure (Fig. 6). Two interactions are found between CARMA1 CARDs. Residue H31 of one CARD interacts with residue E27 through an electrostatic interaction. The conserved residue C28 of each CARD was connected to forms a disulfide bond between helices H1. This disulfide bond induced dimerization might be important in the regulation of CARMA1 oligomerization, which plays a critical role in downstream NF-κB activation. Recent findings suggest that molecules possess a reactive oxygen could activate the formation of inflammasome, through PYD–PYD and CARD–CARD interaction. It can be predicted that reactive oxygen species mediate the formation of CARMA1 complexes [74]. It is noteworthy that the dimer formation of CARMA1 CARD is different from the homotypic interactions of DDs. When compared with the Myddosome assembly, the dimer interface of the resultant CARMA1 CARD dimer would mostly block any homotypic interactions through its type Ia surface. And the CARMA1 dimer also may partly interfere with the homotypic interaction through the type IIIb surface.

Crystal structure of homodimeric NOD1 CARDs shows an extensive interacting area between NOD1 CARDs (Fig. 6). Interestingly, helix H6 (residues from A96 to E106) is swapped with each other. The hinge loop (D95 to D99) between helices H5 and H6 of one NOD1 CARD interacts with the loop (H33 to T37) between helix H1 and helix H2 of another NOD1 CARD through six hydrogen bonds. There is a disulfide bond formed by connecting the residue C39 of each CARD. Usually CARD has a compact fold structure with a hydrophobic core surrounded by six helices. In NOD1 CARD dimer, the helix H6 of one CARD molecule is swapped to provide the hydrophobic residues, L100, W103, and L104, to cover the hydrophobic core of the other CARD [78, 82]. When compared with the Myddosome assembly, the NOD1 dimer would interfere with the homotypic interactions through the type Ib and IIb surface. However, this unique CARD dimer may utilize a novel mechanism to regulate signal complex formation. For example, a recent structural study has found that three disulfide-clinched and domain-swapped NOD1 CARD dimers could form a novel oligomer structure of CARD hexamer (PDB: 4E9 M), although the function of the complex is not clear.

Oligomeric CARD–CARD interaction

A structural study about a complex of RIG-I and MAVS publicized currently [29] has surprisingly revealed a novel mechanism of CARD assembly, which has a helical symmetry similar to that of Myddosome. The tandem CARDs of RIG-I exhibit a tetramer and construct a helical assembly. Three pairs of interaction surface Ia:Ib, IIa:IIb, and IIIa:IIIb mediate assembly of CARD oligomer (Fig. 3). RIG-I CARD1 and CARD2 use the type II (IIa:IIb) interface for intramolecular interaction, and other two interfaces are available for intermolecular interactions. So the types I (Ia:Ib) and III (IIIa:IIIb) interactions are found between the subunits along the tetramer trajectory, and also in the seam region of tetramer formed by the first and forth tandem CARDs.

The structure shows that the surfaces of MAVS CARD (Ia, IIa and IIIa) interact, respectively, with the surfaces (Ib, IIb and IIIb) of RIG-I CARD2 in the RIG-I tandem CARDs complex. In the type I interface, residues S125 and L140 of RIG-I CARD2 interact with residues F16 and W56 of MAVS CARD. Residues R117, I119, D122, M148, R179, and K181 of RIG-I CARD2 and residues D6, R37, R64, and R65 of MAVS CARD are responsible for the type II interface. The interface on the helical seam belongs to the type III interface, in which residues T145, K146, and M149 of RIG-I CARD2 interact with residues R37, D40, R41, A44, and L48 of MAVS CARD.

However, the residues on the surfaces Ib and IIIb of RIG-I CARD2 are different from those on MAVS CARD, which suggests the plasticity of MAVS CARD that permits it to be recruited to RIG-I CARD2 or to interact with MAVS CARD in the subsequently formed RIG-I-MAVS complex, through distinct combinations of surface residues [29] The structural information has led to a novel mechanism of signaling: the tandem CARDs tetramer is termed a 'lock washer', with two ends adjacent to half the thickness of the ring [79]. This 'lock-washer' is served for MAVS-CARD filament formation (Fig. 7), which is important to trigger the downstream signaling.

Filamentous CARD–CARD interaction

Several studies suggest that CARD-containing proteins can form a filament-like structure through CARD-CARD interactions [29, 68]. As mentioned earlier, MAVS CARDs can form a filament when recruited to RIG-I tandem CARDs tetramer. A MAVS CARD interacts with its newest neighbor MAVS CARDs within MAVS filament through the type I, II and III interfaces. The MAVS CARD filament can be seen as a left-handed single-stranded helix with a twist angle of 101° and an axial rise of 5.13Å. CARD-CARD interactions within a filament have both electrostatic and hydrophobic interactions. The intrastrand interactions are from the type IIIa and IIIb patches, in which residues R37, D40, R41, A44, and L48 of one MAVS CARD interact with residues R52, D53, G50, and

L48 of another CARD. The type I interface, including residues R43 and W56, and the type II interface, involving residues R37-R41-R65-R64 in one MAVS CARD and N21-D23-E26 in another CARD, form the interstrand interactions (Fig. 6).

In addition, Bcl-10 CARDs also form a filamentous CARD complex. In the CARMA1/Bcl10/MALT1 (CBM) signalosome, CARMA1 may form a short helical segment through its multiple coiled-coil domains and act as a nucleator to induce the polymerization and elongation of Bcl10 to form a filamentous structure. MALT1 is then added to the filament of Bcl10 by binding to the periphery of Bcl10.

Interestingly, unusually long H1 and H6 of Bcl10 CARD result in its pear-shaped structure that makes it quite different from other DD superfamily structures. CARMA1 possess an extended loop between the helices H3 and H4, which is predicted to be involved in the interaction with Bcl10. CARMA1 and Bcl10 could interact through the type I and II interfaces, between positively charged residues R35, K41, K69, and R72 located on a flexible loop between the helices H3 and H4 of CARMA1, and the negatively charged residues E50, D51, E53, and E54 of Bcl10.

Subsequently, two types of interfaces, intrastand and interstrand, connect the subunits of Bcl10 into a filament. The intrastrand interface involving R36 and E50-D51-E53-E54 is similar to type II interaction surface; in contrast, the interstrand interface containing E30, D39, R42-K44-K45 and R62-K63-R65 is similar to type I interaction face. Based on EM map, the long H1 and H6 helices are crucial in matching the EM density. The resultant Bcl10 filament is a hollow helical assembly with a left-handed four-stranded helical symmetry.

CARD–Ub interaction

CARD–Ubiquitin (Ub) interaction is another trick to regulating signaling. Ub is absolutely important in intracellular signaling pathways. It alters the signaling activities of the target or substrate proteins, in the traditional view, by covalent modification. A mono-Ub or a poly-Ub chain functions when covalently linked to their substrate proteins. K48-linked poly-Ub drives its substrate protein to degradation, in contrast, K63-linked poly-Ub functions to assemble the substrate protein, kinases or other effectors to form a platform for signal transduction [83]. Emerging amounts of evidences have suggested that non-covalent interaction between ubiquitin and the target protein also contributes to the intracellular signaling pathways [79, 84]. Interestingly, Ubiquitin also play a role in regulating the CARD-mediated signaling pathways by binding the CARDs of the innate immune receptors, NOD1, NOD2, RIG-I, and MAD5 [83, 85].

Fig. 8 Crystal structure of the CARD–Ub complexes. Ubiquitin can also interact with CARD-containing proteins, such as NOD1 (PDB: 4JQW) and RIG-I (PDB: 4NQK). The interactions between NOD1 and Ub in the crystals are mainly through hydrogen bonds (by the residues in *red* and *magenta*), a salt bridge (by the residues in *blue*) and the C59-F4 interactions (by the residues in *orange spheres*). Another Ub-binding residue, Y88, identified by a previous NMR study is also shown in *black sticks*. In the crystal structure of the RIG-I-Ub complex, the proximal and distal Ubs bind to RIG-I in different ways. Different faces of RIG-I and different residues of Ub are involved as described in the text. L8, I44, and V70 of Ub are in *red*, while R42 and Q49 are in *green*, and F45, A46, N60, and Q62 are in *yellow*

The mode of Ub binding to NOD1 CARD is unique. NOD1 CARD interacts mainly with the F4 patch of Ub. The Ub-binding interfaces are mainly located on two regions of NOD1. The first one is centered on the residues N36 and T37 on the H1/H2 loop and Q64 on the helix H4 of NOD1 CARD, which interact with the residue E64 of Ub by hydrogen bonding. Q64 of NOD1 CARD also forms a hydrogen bond with K63 of Ub. H3 and H4 helices and the intervening loop region is the second interface that binds T66 of Ub. In addition, E56 and the dimethyl-As modified C59 on the helix H3 of NOD1 CARD interact with K6 and F4 of Ub, respectively (Fig. 8). Although the CARD–Ub interaction is expected to be weak, CARD dimerization or oligomerization could contribute substantially to the binding of poly-Ub, which could be a reason why poly-Ub chains could both regulate NOD1 CARD and RIG-I CARD mediated signaling.

The classical model of RIG-I activation suggests that RIG-I remains in an inactive state in the absence of ligand. A conformational change could be resulted from the covalent conjugation of K63-linked poly-ubiquitin chains, which is the consequence of the binding of E3 ubiquitin ligase tripartite motif 25 (TRIM25) [86]. Ub binds RIG-I tandem CARDs through two types of interactions: Each proximal Ub interacts with two adjacent tandem CARDs and each distal Ub interacts with one tandem CARDs. In the proximal Ub interaction, tandem CARDs interact with Ub through two interfaces. One interacts with the residues L8, I44, and V70 of Ub, another interacts with the residues F45, A46, N60, and Q62 of Ub. In the distal Ub interaction, the distal Ub interacts with tandem CARDs through residues L8-I44-V70, R42, and Q49. Conclusively, noncovalently bound poly-Ub could induce RIG-I oligomerization to form a complex containing four RIG-I and four K63-poly-Ub molecules, subsequently allowing forming a MAVS-CARD filament (Fig. 7).

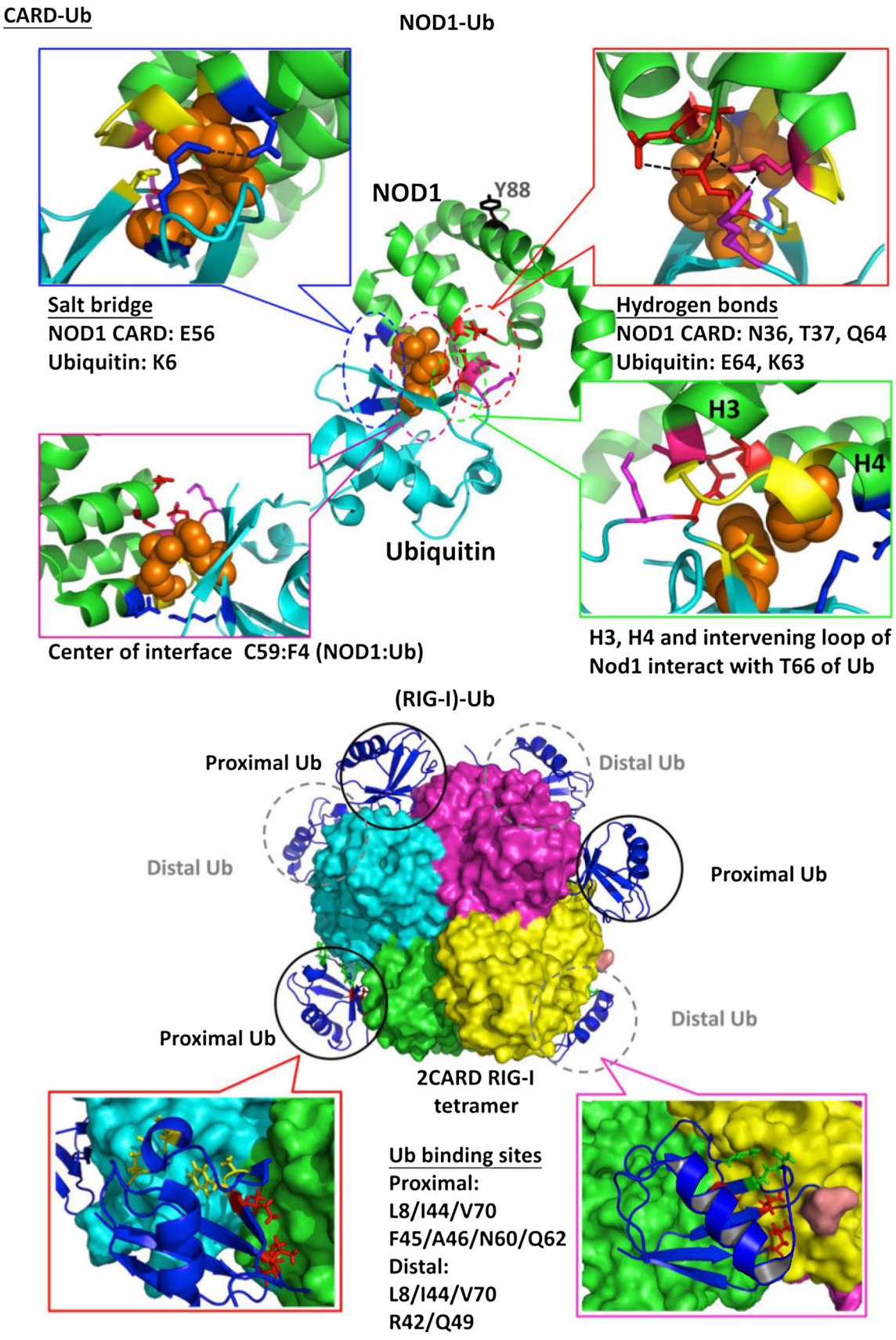
CARD-Ub
NOD1-Ub
NOD1
Y88
Salt bridge
NOD1 CARD: E56
Ubiquitin: K6
Hydrogen bonds
NOD1 CARD: N36, T37, Q64
Ubiquitin: E64, K63
H3
H4
Ubiquitin
Center of interface C59:F4 (NOD1:Ub)
H3, H4 and intervening loop of
Nod1 interact with T66 of Ub
(RIG-I)-Ub
Proximal Ub
Distal Ub
Distal Ub
Proximal Ub
Proximal Ub
Distal Ub
2CARD RIG-I
tetramer
Ub binding sites
Proximal:
L8/I44/V70
F45/A46/N60/Q62
Distal:
L8/I44/V70
R42/Q49

Table 3 The therapeutic drugs targeting CARD-containing proteins

Target	Compound	Class	Principle	Development stage	Ref.
Nod1/2	Curcumin	Polyphenol	Inhibit NOD2 oligomerization	Phase I	[87]
	Parthenolide	Sesquiterpene lactone	Inhibit NOD2 oligomerization, interfere ATPase activity of NLRP3	Phase I	[87]
	Helenalin	Sesquiterpene lactone	Inhibit p65 subunit of NF-κB	Preclinical	[88]
	Pseudopterosin A	Pseudopterosins	Target NOD receptors	Preclinical	[89]
	Docosahexaenoic acid (DHA)	Dietary fatty acids	Prevent NOD2 self-oligomerization	Preclinical	[90]
	Eicosapentaenoic acid (EPA)	Dietary fatty acids	Prevent NOD2 self-oligomerization	Preclinical	[90]
	[a]2-aminobenzimidazole scaffold based series	Benzimidazole	Directly interact with NOD1	Preclinical	[91]
	Purine-2,6-diones (Xanthine, ML146)	Purine	ATP-binding site	Preclinical	[92]
	Noditinib-1	Benzimidazole	Inhibit NOD1-dependent activation of NF-κB and MAPK signaling and also inhibit NOD1-induced IL-8 production	Preclinical	[94]
	GSK217	Aminoquinoline	Inhibit NOD1-dependent activation	Preclinical	[95]
	GSK669	Benzimidazole diamide	Diminish MDP-induced IL-8 release	Preclinical	[96]
	GSK717	Benzimidazole diamide	Compete with the binding of MDP to NOD2	Preclinical	[96]
	Pseudopterosin-based inhibitors	Hydrophenalene-chromium complexes	Compete with the binding of MDP to NOD2	Preclinical	[97]
RIPK2	SB203580	Pyridinyl imidazole	Inhibit RIPK2 and p38	Preclinical	[98]
	Gefitinib	Anilinoquinazolin	Inhibit both RIPK2 tyrosine phosphorilation and MDP-induced cytokine release	Phase III	[99]
	Erlotinib	Quinazoline	Inhibit both RIPK2 tyrosine phosphorilation and MDP-induced cytokine release	Phase IV	[99]
	GSK214	Aminoquinoline	Inhibited RIPK2 enzymatic activity	Preclinical	[96]
cIAP	[a]GDC-0152	Thiadiazole	Disrup protein–protein interaction of IAP proteins	Phase I	[100]
	AEG40826/HGS1029	Hydrochloride salt	Inhibits the biological activity of IAP proteins	Phase I	[102]
	[a]LBW242	Smac mimetic compound	Destruction of cIAP1	Preclinical	[103]
	BV6	Structure-based design	Auto-ubiquitination and proteasomal degradation of cIAP proteins	Preclinical	[104]
	Compound A	Smac mimetic compound	Promote the degradation of cIAP1 and cIAP2	Preclinical	[105]
	Compound 3 (SMC3)	Smac mimetic compound	Activates apoptosis and NF-κB through autocrine TNF	Preclinical	[106]
	Compound 11	Bicyclic Smac mimetic	High binding affinities to IAP	Preclinical	[107]

Table 3 continued

Target	Compound	Class	Principle	Development stage	Ref.
cIAP	[a]Compound 8	Smac mimetic compound	Bind to IAP	Phase I	[107]
	[a]SM-122, SM-164	Smac mimetic compound	Bind to IAP	Preclinical	[107]
	[a]LCL-161	Smac mimetic compound	Degradation of cIAP1 and cleavage of caspase 3	Phase I	[108]
	AT-406	Smac mimetic compound	Effectively target xIAP and cIAP1/2	Phase I	[109]
	[a]Bestatin methyl ester	Small molecule	Destabilize cellular cIAP1	Preclinical	[110]
	[a]Bestatin-actinonin hybrid, HAB-5(30b)	Small molecule	Destabilize cellular cIAP1	Preclinical	[110]
	CS-3	Small molecule	Trigger cIAP1 and cIAP2 degradation and NF-κB activation	Preclinical	[101]
	Ro106-9920	Tetrazolopyridazine-phenylsulfoxide	Inhibit an ubiquitination activity	Preclinical	[111]
Apoptosome activity	Poly-L-glutamic acid-peptoid 1 (PGA-peptoid 1)	*N*-alquilglycine (Apaf-1 inhibitor)	Enhance the antiapoptotic activity	Preclinical	[112]
	QM31	Cytoprotective agent	Inhibite apoptosome activation and mitochondrial outermembrane permeabilization	Preclinical	[113]
	[b]A2.Y24G33 (YIMDHMISDG)	Peptide	Inhibit apoptosome activity (from Apaf1)	Preclinical	[93]
	[b]A3.F34V43 (YGG-FLTISEEEKV)	Peptide	Inhibit apoptosome activity (from Apaf1)	Preclinical	[93]
	[b]N4.Q64V75 (YGG-VVLDKLHGQ)	Peptide	Inhibit apoptosome activity (from NOD1)	Preclinical	[93]
	[b]Phg4 Cyclo-CSWFEASYC	Peptide	Inhibit apoptosome activity (from Apaf1)	Preclinical	[93]
	[b]Np1.L1379R1392 (LHFVDQYREQLIAR)	Peptide	Inhibit apoptosome activity (from NLRP1)	Preclinical	[93]
	[b]As1.D116R125 (YGG-DQHRAALIAR)	Peptide	Inhibit apoptosome activity (from ASC)	Preclinical	[93]
	[b]As4.N155T166 (NPSKMRKLFSFT)	Peptide	Inhibit apoptosome activity (from ASC)	Preclinical	[93]
Caspase-1	VX-765	Small molecule	Reducing the release of IL-1β and IL-18	Phase III	[114]
	Ac-YVAD-CHO	Peptide	Reducing the release of IL-1β and IL-18	Preclinical	[115]
	Ritonavir	Peptidomimetic agent	Reduce active IL-18 level	Phase II	[116]
Inflammasome blockers	N-acetyl cycteine (NAC)	Antioxidant	Block NLRP3 inflammasome activation	Phase I	[117]
	Glyburide	Sulphonylurea	Block IL-1β processing	Preclinical	[118]
	Cytokine release inhibitory drug 3 (CRID3 or CP-456,773)	Diarylsulphonylurea	Inhibit the post-translational processing of IL-1β by targeting Glutathione S-Transferase Omega 1 (GSTO1)	Preclinical	[119]
	Acetylate 18-mer peptide (acaly18: acetyl-ALYDKGYTSKEQKDCVGI)	Peptide	Activate NLRP3 inflammasome	Preclinical	[120]

[a] Compounds directly interact with CARDs proteins

[b] Designed peptide based on the structure of CARD domain

Conclusions and perspectives

CARD, containing 90 to 100 amino acids, is a small protein–protein interaction module with the 10–20 % amino acid sequence identity. CARD is the second largest subfamily of the DD superfamily. CARD-CARD interactions play a pivotal role in the signaling complexes formation. The number and function of CARD-containing proteins have dramatically expanded recently. Unravel the mechanism of CARD-containing signaling complex formation in apoptosis can contribute to the treatment of apoptosis-related diseases, such as cancer and inflammatory diseases. CARD-containing proteins can be subdivided into several subgroups based on their structures, functions, roles and the signaling complexes they are involved. It shows that CARD-containing proteins with similar overall domain features may have similar functions or roles.

CARDs possess the polarized surface with the acidic and basic patches. Although they share a low sequence identity, CARDs adopt the structure features of the DD superfamily members with a six-helical bundle, but helix H1 tends to bend or break into separated H1a and H1b helices by a kink in H1. The homotypic interactions between CARDs occur through their acidic and basic surfaces, which can be divided into three different types, type I, II and III.

To understand the relevance between the mechanism of downstream signaling pathways and complexes formation, it is important to study the oligomerization way of CARD-containing proteins. Dimerization is a simple oligomerization way, which contains hetero- and homo-dimerization. Apaf-1 and Caspase-9 form a heterodimeric structure through the type I interface. In contrast, the homodimeric structures, such as a dimer of NOD1 or CARMA1, are assembled with an intermolecular disulfide bond. The disulfide bond mediated dimeric structure formation is not observed in other members of the DD superfamily. A novel oligomerization way seems to implicate a novel regulatory mechanism in intracellular signaling pathways.

However, recent publications represent a different oligomerization way to form signaling complexes that arouses a brand new outlook on the studying of apoptosis, inflammatory and immune signaling. The new discovered mechanism could show us how one of our immune systems could detect the invading foreign pathogen in order to protect us from the attacks.

A recent study of RIG-I [29] suggests that RIG-I CARDs can form a tetramer of tandem CARDs, which can form a stage for MAVS CARDs to build a left-handed single-stranded helical filament. Previous studies indicate that the first CARD of RIG-I tightly interacts with second CARD of RIG-I to stabilize the tandem CARDs tetramer. In addition, it also suggests that ubiquitin chains might surround around tandem CARDs tetramer and stabilize the tetramer, in turn allowing the assembly of a MAVS-CARD filament.

Both CARMA1 and RIG-I are located in one end of filament-like structure and act as a nucleator or platform, leading to the directional elongation of Bcl10 and MAVS, respectively. In this case, the assembly of CARD oligomerization is in an analogous manner to the formation of cytoskeleton. However, these structures are formed using mostly CARD only protein. In a physiological system, how the full-length proteins assemble to form a long filament surrounded with other domains and why we need such a huge aggregate for immune responses require further investigations. In addition, ubiquitin could stabilize the structure of CARD oligomer for the assembly of the signaling complex. To unravel the mechanisms that control the assembly and disassembly of the signaling complexes is also importance. Furthermore, this review shows versatile roles of CARDs. Limited structures of the CARD complexes, however, are available for elucidating the signaling mechanism. Whether other CARD-containing signaling complexes utilize similar mechanisms in complex assembly, how CARDs form a specific complex, how CARDs form a CARD-specific complex, what the difference is between CARD-CARD interaction and DD-DD interaction, and ultimately whether we could manipulate the signaling complexes to control the signaling pathways also require further investigations.

Current status of therapeutic drugs targeting CARD-containing proteins

CARD-mediated protein–protein interaction is one of the major contributors for the signaling complex formation that leads to apoptosis or immune responses. Hence operation of the signal transduction by the drugs targeting CARDs or CARD-containing proteins has significant therapeutic potential. Several therapeutic drugs targeting CARD-containing proteins or related complexes, including NOD1/2, RIPK2, cIAP, apoptosome, and inflammasome, had been reported (Table 3).

Several natural compounds that have anti-inflammatory or antitumor properties could be the inhibitor of NOD1/2. For example, curcumin and parthenolide could inhibit NOD2 oilgomerization and suppress NF-κB activation and IL-8 expression [87]; Helenalin also suppresses NF-κB activation by inhibiting the p65 subunit of NF-κB [88], while pseudopterosin A is related to anti-inflammatory actions through targeting NOD receptors [89]; Dietary fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are indicated to be able to suppress NF-κB activation and IL-8 expression by preventing NOD2

self-oligomerization [90]. In addition, several NOD1/2 inhibitors, which would affect NOD-mediated signaling, had been screened out from molecular libraries [91–97].

RIPK2 is also a key player in the nodosome signaling pathway, which mediates cytokine release in inflammation; hence RIPK2 is also a drug target. SB203580 [98] and GSK214 [96] are suggested to inhibit RIPK2 enzymatic activity. Gefitinib and erlotinib, which are executing phase III and IV clinical trials, respectively, to treat non-small cell lung cancer, could inhibit both RIPK2-mediated tyrosine phosphorylation and MDP-induced cytokine release [99].

Unlike the strategy through anti-inflammation or suppressing NF-κB activation to suppress tumor growth, inducing apoptosis in cancer cells is another therapeutic approach to treat cancer. An idea to inhibit IAPs has been carried out. Many Smac mimetics that inhibit IAPs and drugs that promote IAP degradation had been reported [100–111]. Most of them, except the Smac mimetic compound 3 (SMC3) that could activate NF-κB through autocrine TNF, promote apoptosis through degrading or directly binding to IAP proteins.

As mentioned earlier that apoptosis plays a critical role in maintaining the homeostasis of biological organisms. Dysregulation of apoptosis could result in serious diseases. For example, ischemia, heart failure, neurodegeneration, inflammation, osteoarthritis, AIDS, bacterial infection, allograft rejection and graft versus host disease, type I diabetes and trauma are related to excessive apoptosis. Some drugs that could block the function of apoptosome were developed in order to inhibit caspase activation and subsequent apoptosis, which could be the way to treat these diseases [93, 112, 113].

However, to treat diseases such as inflammatory diseases and autoimmune disorders, one approach is to inhibit the production of mature IL-1β by inhibiting caspase-1 activity or the function of inflammasomes, which have critical roles in the innate immunity. Several inhibitors of caspase-1, which plays a pivotal role in the pro-inflammatory reactions, have been reported to reduce the release of IL-1β and IL-18 [114–116]. Several drugs also have been reported to manipulate the function of inflammasome [117–120]. Most of them are still in preclinical stages of drug development.

Most of these drugs were screened out based on examining the effects on enzymatic activity or cytokines release. Only few of them are directly targeting CARDs. There are several peptides designed based on the structures of CARDs or the CARD complexes. And some compounds that could directly interact with CARDs have also been developed (Table 3). Therapeutic drugs developed from examining downstream enzymatic activity that block apoptosis or inflammatory signaling may also interrupt other physiological signaling pathways. Hence, it is important to develop more drugs targeting on specific CARD–CARD interactions.

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