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REVIEW

NF-κB signaling pathways regulated by CARMA family of scaffold proteins

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The NF-κB family of transcription factors plays a crucial role in cell activation, survival and proliferation. Its aberrant activity results in cancer, immunodeficiency or autoimmune disorders. Over the past two decades, tremendous progress has been made in our understanding of the signals that regulate NF-κB activation, especially how scaffold proteins link different receptors to the NF-κB-activating complex, the IκB kinase complex. The growing number of these scaffolds underscores the complexity of the signaling networks in different cell types. In this review, we discuss the role of scaffold molecules in signaling cascades induced by stimulation of antigen receptors, G-protein-coupled receptors and C-type Lectin receptors, resulting in NF-κB activation. Especially, we focus on the family of Caspase recruitment domain (CARD)-containing proteins known as CARMA and their function in activation of NF-κB, as well as the link of these scaffolds to the development of various neoplastic diseases through regulation of NF-κB.

***Keywords*:** CARMA1; CARMA2; CARMA3; CARD9; Bcl10; NF-κB; IKK; NEMO

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Introduction

Scaffold proteins are defined as molecules that bind to at least two other signaling proteins [1]. Scaffold proteins typically do not posses any enzymatic or transcriptional activity, but they have the ability to assemble various combinations of multi-protein complexes, necessary for integration of signals and selective transmission of information from the surface receptors [1, 2]. In most cases, scaffolds help to localize signaling molecules to specific parts of cell. They also serve as platforms for assembling enzymes and their substrates, restraining the nonspecific access of enzymes to unwanted substrates and protecting from undesirable cellular effects [3]. Some scaffold proteins can have other functions, like coordination of the positive and negative feedback signals or protection of activated proteins from inactivation [1]. Interestingly, scaffold proteins may exhibit distinct functions under different physiological conditions [4]. Also, multiple scaffold/receptor complexes may exist simultaneously, directing both overlapping and distinct cellular events [2].

To date, a large number of scaffolds have been shown to play a crucial role in activation of the nuclear factor κB (NF-κB) following stimulation of different receptors [5-9]. NF-κB is a family of transcription factors that control cell activation, proliferation and survival. Its activity is tightly regulated by interaction with inhibitory proteins, IκBs, which mask the nuclear localization sequence of NF-κB subunits, thereby sequestering NF-κB in cytoplasm. Upon stimulation, IκB is phosphorylated by the IκB kinase (IKK) complex, followed with ubiquitination-dependent degradation by the 26S proteasome complex. Therefore, NF-κB can be translocated into the nucleus and initiate specific target gene transcription [5, 10]. In spite of the tremendous progress in the NF-κB signaling field, the signaling cascades connecting different receptors to the IKK complex remain to be fully determined (Figure 1). Previous studies have revealed the tissue-specific and stimulus-specific roles of many scaffold proteins in regulating NF-κB signaling networks [7, 8, 11, 12]. Among these scaffold proteins, a family of Caspase recruitment domain (CARD)-containing scaffold proteins, known as CARD- and membrane-associated guanylate kinase-like domain-containing protein (CARMA), plays critical roles in recruitment and activation of IKK [12, 13]. In this

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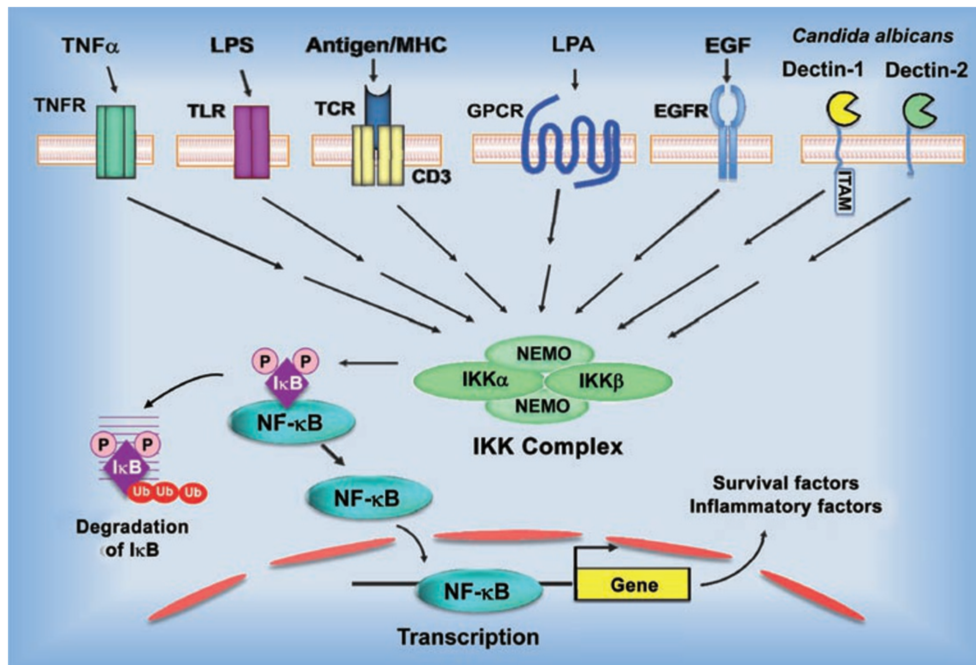


Figure 1 Model of NF-κB activation by the canonical pathway. Stimulation of the surface receptors by different inducers initiates several proximal signaling events resulting in activation of the IκB kinase (IKK) complex, composed of two kinases, IKKα and IKKβ, and the regulatory subunit NF-κB-essential modulator (NEMO). IKK phosphorylates inhibitor of κB (IκB), which leads to its ubiquitination and subsequent degradation. NF-κB is then translocated into nuclei and initiates the target gene transcription. TNFR – tumor necrosis factor receptor; TLR – toll like receptor, LPS – lipopolysaccharide; TCR – T-cell receptor; GPCR – G protein-coupled receptors; LPA - lysophosphatidic acid; EGFR - epidermal growth factor receptor; ITAM - immunoreceptor tyrosine-based activation motif; Ub - ubiquitin.

article, we discuss the role of CARMA family members and other related CARD-containing scaffold proteins in regulation of NF-κB activation in response to signals induced by the various cell surface receptors.

CARMA family of scaffold proteins

The CARMA family is conserved among species and has three members, CARMA1, CARMA2, and CARMA3 that are encoded by three different genes [14-16]. They all contain an N-terminal CARD domain, followed with a coiled-coil domain (C-C), a PDZ domain, an SH3 domain, and a Guanylate Kinase-like (GUK) domain in the C-terminus. The structural module of PDZ-SH3-GUK is also called Membrane-associated GUK (MAGUK) domain. CARMA family members were initially identified based on their CARD domain by bioinformatics approaches and named CARD11 (known as CARMA1 or Bimp3), CARD14 (known as CARMA2 or Bimp2), and CARD10 (known as CARMA3 or Bimp1) [14, 16, 17]. CARMA1, CARMA2, and CARMA3 share high degree of sequence and structural homology (Figure 2), but they exhibit a distinct tissue distribution pattern. Original studies suggest that CARMA1 is primary expressed in hematopoietic tissues such as spleen, thymus, and peripheral blood leukocyte; CARMA2 is expressed in placenta; and CARMA3 is expressed in a broad range of tissues but not in hematopoietic cells [14-17]. We have analyzed the mRNA microarray data, which were generated from 353 human tissue samples [18] and deposited in the databank (Oncomine 4.4; www.oncomine.org). This analysis revealed that CARMA2 is mainly expressed in mucosa tissues (Figure 3), although the expression pattern of CARMA1 and CARMA3 is consistent with the initial studies [14-17]. This distinct tissue distribution suggests that CARMA family members may have the same function to activate downstream signaling events and play similar roles in different cell types. Indeed, ectopic expression of CARMA family members induces potent activation of NF-κB in most of cell lines, as well as primary cells [6, 15, 19]. The overexpressed CARMA1 forms a complex with two downstream signaling molecules, Bcl10 (B-cell lymphoma protein 10), another CARD-containing scaffold protein, and MALT1 (Mucosa-associated lymphoid tissue lymphoma translocation protein 1), a caspase-like protein [20]. Previous

studies demonstrate that signal-dependent formation of the CARMA1-Bcl10-MALT1 complex (commonly known as the CBM complex) recruits downstream signaling components, leading to the activation of NF-κB [20-22].

CBM proteins in antigen receptor signaling

By inducing somatic mutations in Jurkat T cells, our lab obtained a cell line that lacks CARMA1 protein expression. Using this CARMA1-deficient cells, we demonstrated that CARMA1 is required for the T cell receptor (TCR)-induced NF-κB activity [23]. Independent studies using dominant negative mutants of CARMA1 [24] or small interfering RNA targeting CARMA1 [25] also revealed the crucial role of CARMA1 in NF-κB activation following TCR ligation. Finally, the gene-targeting experiments in mouse have further confirmed that CARMA1 is essential for antigen receptor-induced NF-κB and JNK activation, but not ERK or p38 activation [26-28]. Although the development and survival of mature B and T cells are not significantly affected by CARMA1 deficiency, the signal-induced proliferation of mature cells is severely impaired [26, 27]. Similar defects are observed in Bcl10-deficient lymphocytes [29].

Bcl10 was identified by functional cloning from mucosa-associated lymphoid tissue (MALT) lymphoma cells [30, 31] and by bioinformatics approaches as a CARD-containing protein [32-34]. Genetic studies using Bcl10-deficient mice have revealed that Bcl10 is an essential component in the T cell receptor (TCR)- and B cell receptor (BCR)-induced NF-κB activation, and functions downstream of PKC [29, 35]. Bcl10 contains an N-terminal CARD domain and a C-terminal Ser/Thr-rich domain (Figure 2). The CARD domain of Bcl10 is responsible for its association with CARMA1 and TCR-induced oligomerization [36, 37]. Bcl10 oligomers can function as scaffolds for the IKK and Jun N-terminal kinase (JNK) pathways by recruiting and assembling signaling complexes containing kinases and their substrates [12]. In this case, oligomerized Bcl10 associates with JNK2 and its upstream kinases, MKK7 and TAK1, in stimulated Jurkat T cells [38]. The high molecular weight complex of oligomerized Bcl10 was also found to regulate TCR-induced actin polymerization [37]. Interestingly, Bcl10-dependent actin polymerization has a significant impact on phagocytosis in monocytes, but CARMA1 and MALT1 are not involved in this process [39, 40].

Similarly to Bcl10, MALT1 (also known as Paracaspase) was first identified by genetic cloning from MALT lymphoma patient samples [41] and bioinformatic approach [42]. *Malt1* gene is localized in a break point of chromosome 18q21 and t(11;18)(q21;q21) generates API2-MALT1 fusion protein, whereas t(14;18)(q32;q21) juxtaposes *Malt1* gene to the immunoglobulin locus and upregulates its expression [41, 43, 44]. Although transient transfection of wild type MALT1 does not significantly activate NF-κB, overexpression of its oncogenic form potently activates NF-κB *in vitro* [42, 45]. Consistent with these results, transgenic mice expressing Eμ-API2-MALT1 have elevated NF-κB activity [46]. On the other hand, genetic inactivation of *Malt1* gene in mice impairs TCR-induced NF-κB activation [47, 48]. However, there are some discrepancies about the role of MALT1 in BCR-induced NF-κB activation in two mouse models. One study suggests that total NF-κB activity is significantly reduced in MALT1-deficient B cells [47], whereas another gene-targeting study shows that NF-κB activation is almost not affected by MALT1 deficiency upon BCR stimulation [48]. Surprisingly, further work has revealed that the activation of c-Rel isoform of NF-κB is more severely impaired than other isoforms, such as RelA (known as p65) [49]. Thus, it remains to be de-

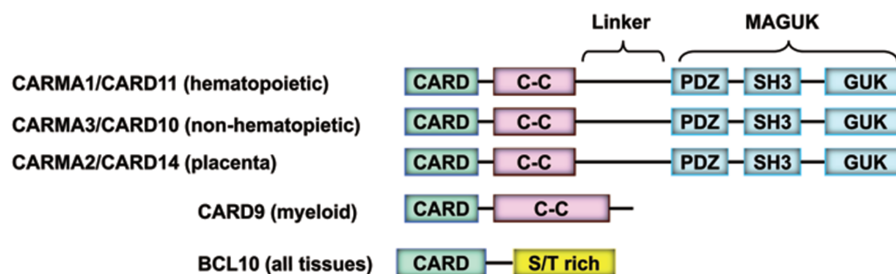
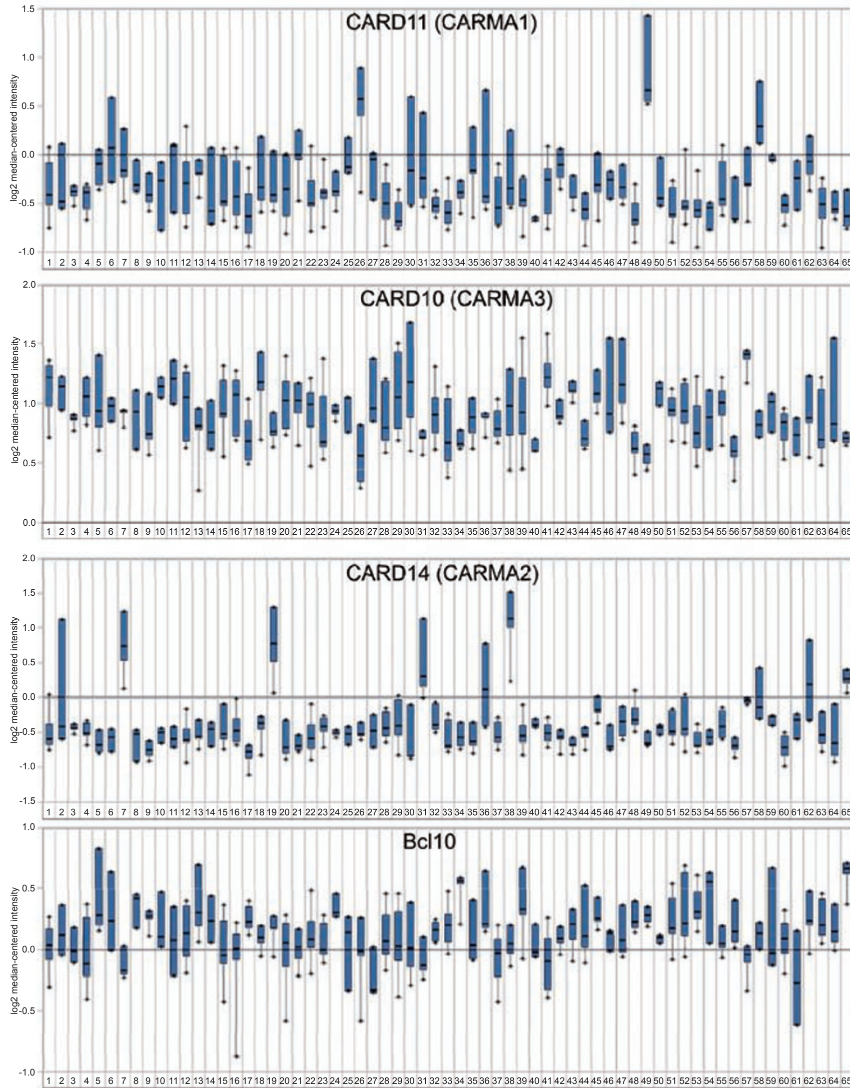


Figure 2 Structures of CARMA1-3, CARD9, Bcl10 and their tissue distribution. CARMA - caspase recruitment domain (CARD)- and membrane-associated guanylate kinase-like domain-containing protein; Bcl10 - B-cell lymphoma 10; CARD - caspase-recruitment domain; C-C - coiled-coil domain; MAGUK - membrane-associated guanylate kinase (GuK)-like domain; S/T rich – Ser/Thr rich domain.



Legend

1. Accumbens Nucleus
2. Adipose Tissue
3. Adrenal Cortex
4. Amygdala
5. Bone Marrow
6. Bronchus
7. Buccal Mucosa
8. Gastric Cardia
9. Cardiac Atrium
10. Cardiac Ventricle
11. Cecum
12. Cerebellum
13. Cervix Uteri
14. Coronary Artery
15. Corpus Callosum
16. Cerebral Cortex
17. Dorsal Root Ganglion
18. Endometrium
19. Esophagus
20. Frontal Lobe
21. Fundus of the Stomach
22. Hippocampus
23. Hypothalamus
24. Liver
25. Lung
26. Lymph Node
27. Mammary Gland
28. Medulla Oblongata
29. Mesencephalon
30. Myometrium
31. Nipple
32. Nodose Ganglion
33. Occipital Lobe
34. Omental Adipose Tissue
35. Ovary
36. Papilla of the Tongue
37. Parietal Lobe
38. Pharyngeal Mucosa
39. Pituitary Gland
40. Prostate Gland
41. Putamen
42. Pylorus
43. Renal Cortex
44. Renal Medulla
45. Salivary Gland
46. Saphenous Vein
47. Skeletal Muscle Tissue
48. Spinal Cord
49. Spleen
50. Subcutaneous Adipose Tissue
51. Substantia Nigra
52. Subthalamic Nucleus
53. Temporal Lobe
54. Testis
55. Thalamus
56. Thyroid Gland
57. Tongue
58. Tonsil
59. Trachea
60. Trigeminal Ganglion
61. Urethra
62. Vagina
63. Ventral Tegmentum
64. Vestibular Nucleus
65. Vulva

Figure 3 Expression patterns of CARMA1, CARMA2, CARMA3 and Bcl10 in 65 different human tissues. Microarray data from the public database were analysed using Oncomine 4.4 tools (www.oncomine.org). Log2 median intensity is shown on Y-axis.

termined why MALT1 deficiency has more significant impact for the activation of c-Rel than other isoforms of NF-κB in B cells.

The initial characterization of MALT1 did not reveal its protease activity [42, 45], and MALT1 has been suggested to serve as an E3 ligase for the regulatory subunit of the IKK complex [50]. Later, it has been shown that MALT1, through its paracaspase domain but in a protease-independent manner, controls caspase-8 (CASP8) activation, leading to activation of NF-κB and production of IL-2 [51]. However, an independent study indicates that MALT1 has an arginine-directed protease activity that is induced following TCR stimulation [52]. The activated MALT1 can process Bcl10 and regulates TCR-induced cell adhesion to fibronectin [52]. This study suggests that MALT1 protease activity is essential for the optimal activation of NF-κB, although it remains to be determined how this activity links to NF-κB. The involvement of MALT1-dependent CASP8 activation in TCR-induced NF-κB activity is consistent with the previous finding that CASP8 is involved in positive regulation of NF-κB activity [53, 54]. In addition, CASP8 and c-FLIP are recruited into lipid rafts following with rapid cleavage of c-FLIP at the CASP8 cleavage site [55]. Together, these studies suggest that an uncharacterized signaling cascade may connect MALT1, CASP8, and c-FLIP to NF-κB activation. Further work is required to address this possible connection.

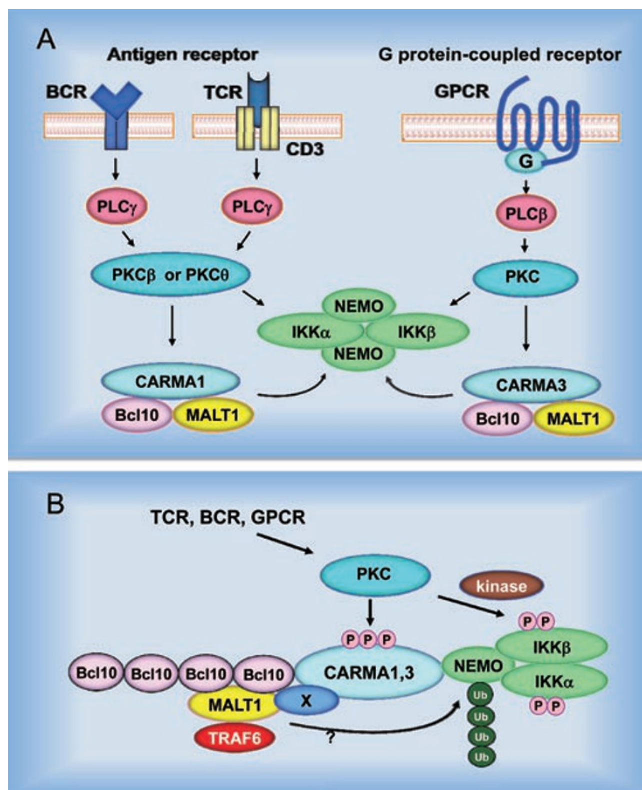


Figure 4 Schematic model of the B-cell receptor (BCR)-, T-cell receptor (TCR) and G protein-coupled receptor-induced NF-κB activation. **(A)** Stimulation of the surface receptor initiates several proximal signaling events that lead to activation of phospholipase C (PLC) and protein kinase C (PKC). The activated PKC phosphorylates CARMA and enables CARMA to associate with the downstream signaling components, Bcl10 and MALT1. Formation of the CBM complex leads to activation of the IKK complex. **(B)** The CBM complex cooperates with tumor necrosis factor receptor-associated factor 6 (TRAF6) and possibly some unidentified protein (X) to mediate ubiquitination of NEMO. The IKK complex is also phosphorylated in a PKC-dependent manner and both modifications are essential for the IKK kinase activity.

The CBM complex in natural killer and mast cells

Besides its critical role in the TCR and BCR signaling, the CBM complex mediates NF-κB activation induced by the multiple immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors [8, 56, 57]. In natural killer (NK) cells, activation of the ITAM-coupled receptors leads to CARMA1-, Bcl10- and MALT1-dependent induction of NF-κB and production of proinflammatory cytokines [56, 57]. However, the CBM complex is dispensable for NK cell–mediated target cell killing [8, 57]. Furthermore, activation of the Fc epsilon receptor I (FcεRI) on mast cells also engages Bcl10 and MALT1 to activate NF-κB [58, 59]. The Bcl10-Malt1 complex promotes IL-6 and tumor necrosis factor (TNF)-α release, which are independent of degranulation and leukotriene secretion [58]. However, it remains to be determined whether CARMA1 is also involved in FcεR-induced signaling events. Together, these studies indicate that CBM proteins play a critical role in multiple receptor signaling pathways.

The mechanism of T cell receptor-induced CARMA1 activation

Although the molecular mechanism is not fully understand, CARMA1 is recruited into the immunological synapse (also known as lipid raft microdomain) upon TCR stimulation [24, 60, 61]. This recruitment seems to be dependent on at least two steps. First, CARMA1 needs to be localized to the cytoplasmic membrane [24] and the point mutation in the MAGUK domain of CARMA1 (Leu808 replaced with Pro) impairs its membrane localization and recruitment to the immunological synapse [61]. This result is consistent with the function of

MAGUK domain that is believed to link proteins to the cytoplasmic membrane [62]. The second step for CARMA1 recruitment to the immunological synapse seems to be dependent on its inducible interaction with an adaptor protein known as ADAP (adhesion- and degranulation-promoting adapter protein). Upon TCR engagement, ADAP and CARMA1 are recruited into the immunological synapse, and ADAP deficiency results in impaired CARMA1 translocation leading to reduced NF-κB activation [63]. However, ADAP is not a membrane protein and it is unlikely that ADAP anchors the MAGUK domain of CARMA1 to the cytoplasmic membrane. Therefore, another unknown protein may be required for anchoring CARMA1 to the cytoplasmic membrane.

Upon TCR ligation, CARMA1 physically associates with protein kinase C theta (PKCθ), and this association is dependent on the linker region (residues 432–671) between the C-C and PDZ domains of CARMA1 (Figure 2) [61]. The linker region is phosphorylated by PKCθ (or PKCβ in B cells) in a signal-dependent manner (Figure 4A) [19, 64] and two putative PKC phosphorylation sites, Ser552 and Ser645 (in mouse: Ser564 and Ser657), were identified and confirmed in the linker region of human CARMA1 [19, 64, 65]. The inducible phosphorylation of CARMA1 by PKC results in conformational changes that enable CARMA1 to associate with its downstream signaling components [19, 64, 66].

The linker region of CARMA1 also contains multiple non-PKC sites and other kinases may be involved in its phosphorylation and regulation. Indeed, PDK1, AKT, TAK1, IKKβ, CK1α, and HPK1 have been shown to associate with or may phosphorylate CARMA1 [67-71]. Moreover, some kinases may contribute to CARMA1 phosphorylation outside the linker region. Consistent with this notion, calmodulin-dependent protein kinase II (CaMKII) is recruited to the immunological synapse following TCR stimulation, and phosphorylates CARMA1 on Ser109 [72]. Mutation of Ser109 to Ala residue in CARMA1 impairs its biological function [72]. Once activated, CARMA1 recruits the IKK complex [60, 61] and other signaling molecules (Table 1) [63, 67-69, 73], including Bcl10 and its pre-associated partner MALT1 [60, 61, 74]. TCR-induced formation of the CARMA1-Bcl10-MALT1 complex is critical for IKK activation. It is conceivable that CBM functions as a molecular platform to assemble IKK with its direct activators [68, 75].

The mechanism of CARMA1-dependent activation of the IKK complex

NF-κB is activated by at least two signaling pathways: the canonical pathway and the non-canonical pathway. These two pathways utilize different IKK complexes to activate downstream signaling. The canonical pathway is activated by the IKK complex containing two catalytic subunits, IKKα and IKKβ, and the regulatory subunit, NF-κB-essential Modulator (NEMO, also known as IKKγ) [76-78], which phosphorylates IκBs and induces their degradation. In contrast, the non-canonical pathway is activated by the IKK complex containing IKKα homodimer, which phosphorylates p100 leading to proteolytic processing of p100 into p52 [10]. Our previous

Table 1 CARMA1-interacting proteins

INTERACTING PROTEIN	BIOLOGICAL FUNCTION	REFERENCE
ADAP	adaptor protein, signal transduction	Medeiros, 2007 (63)
AKT	kinase	Narayan, 2006 (69)
β-arrestins	adaptor protein, signal transduction	Sun, 2008 (6)
Bcl10	scaffold, signal transduction	Bertin, 2001 (14); Gaide, 2001 (15)
CaMKII	kinase	Ishiguro, 2006 (72)
Cbl-b	E3 ligase	Kojo, 2009 (104)
CK1α	kinase	Bidere, 2009 (70)
COP9 (CNS5)	adaptor protein, signal transduction	Welteke, 2009 (106)
HPK1	kinase	Brenner, 2009 (71)
IKKβ	kinase	Wegener, 2006 (21); Shinohara, 2007 (65)
MALT1	paracaspase, protease	Che, 2004 (74); Wegener, 2006 (21)
NEMO	regulatory protein	Stilo, 2004 (92); Shambharkar, 2007 (79)
PDK1	kinase	Lee, 2005 (67)
PKCθ	kinase	Wang, 2004 (61); Matsumoto, 2005 (19)
TAK1	kinase	Shinohara, 2005 (68)

study indicates that CARMA1 is involved in regulating the canonical pathway [79]. Although there is no report implicating a role of CARMA1 or Bcl10 in the non-canonical pathway, one study suggests that MALT1 may be required for BAFF receptor-induced p100 processing [80].

The activation of the IKK complex is dependent on the signal-induced phosphorylation of IKKα and IKKβ on two Ser residues (Ser176/180 for IKKα and Ser177/181 for IKKβ) within the activation loop of the kinase domain [81, 82]. Although many kinases were shown to phosphorylate IKKs when overexpressed in cells, the genetic evidence indicates that two MAP3K kinases, TAK1 (TGFβ–activated kinase 1) [83-86] and MEKK3 (Mitogen-activated Protein Kinase Kinase Kinase 3) [87, 88], are involved in phosphorylation of these two Ser residues in IKKα and IKKβ. Accumulating evidence indicates that TAK1 [83-86] and MEKK3 [88-91] are involved in TNFR-, IL-1βR-, TLR-, and TCR-induced IKK phosphorylation and activation. However, CARMA1 does not control this signal-induced phosphorylation of IKK, instead, CARMA1 directly associates with NEMO [92], and modulates the polyubiquitination of NEMO upon TCR stimulation (Figure 4B) [79].

Although NEMO has no catalytic activity, it is required for the kinase activity of the IKK complex in the canonical NF-κB pathway. It has been shown that NEMO can specifically recognize Lysine 63 (K63)-linked polyubiquitin chains [93-95], and becomes polyubiquitinated upon activation of NF-κB signaling cascades [50, 96]. These two properties of NEMO seem to be required for activation of the IKK complex. It is also possible that K63-linked NEMO polyubiquitination allows NEMO oligomerization through cross-recognition by its own ubiquitin-binding domain and leads to generation of the high molecular weight IKK complex [97]. Interestingly, several studies suggest that K63-linked polyubiquitination of NF-κB signaling components, such as RIP1 [93, 98], IRAK [99], Bcl10 [95], and MALT1 [100], might be recognized by NEMO following stimulation of different receptors. Indeed, it has been shown that TCR ligation leads to K63-linked polyubiquitination of Bcl10 at Lys31 and Lys63 in the CARD domain, and this polyubiquitin chain can be recognized by NEMO [95]. Thus, polyubiquitinated Bcl10 may be involved in a signal-dependent IKK redistribution and possibly activation [36, 95]. Also, all these results suggest that NEMO might be responsible for the recruitment of the IKK complex to the specific subcellular location and/or to the specific signaling complex. However, how NEMO is involved in activation of the IKK complex still remains to be determined.

The CBM complex most likely serves as a molecular platform to recruit signaling components responsible for the K63-linked polyubiquitination of NEMO [50]. Interestingly, although IKKβ phosphorylation is not defective in stimulated CARMA1- or Bcl10-deficient cells, the kinase activity of IKK is completely abolished [79], suggesting that the phosphorylation of IKK is not sufficient to induce its kinase activity. Indeed, activation of the IKK complex is not only dependent on IKK phosphorylation but also on CARMA1-dependent NEMO modification [79]. Initially, the physical interaction of NEMO with CARMA family members, was identified by the yeast two-hybrid screening and confirmed by co-precipitation experiments in mammalian cells [92]. Our later study has revealed that NEMO is polyubiquitinated in a CARMA1-dependent manner upon TCR engagement [79], although the mechanism by which CARMA1 regulates NEMO polyubiquitination is not fully defined. One study suggests that the CBM complex recruits TRAF6, and TRAF6 induces K63-linked polyubiquitination of NEMO upon TCR stimulation [75]. Alternatively, MALT1 may induce NEMO ubiquitination at the Lys399 (Lys392 in mice) residue, because NEMO variant with K399R mutation has been shown to interfere with NF-κB activation [50]. However, mice expressing the NEMO-K392R mutant are not defective in antigen receptor-induced responses [101]. Therefore, it remains to be determined whether MALT1-dependent polyubiquitination of NEMO is functionally important for TCR-induced responses. In addition, although TRAF6 has been suggested to mediate TCR-induced polyubiquitination of the IKK complex [75], T cell-specific deletion of TRAF6 does not impair TCR-induced NF-κB activation [102], suggesting that either there is a redundant mechanism for TRAF6-mediated activation of IKK or TRAF6 is not involved in the activation of IKK (Figure 4B). Because CARMA1-dependent NEMO polyubiquitination is required for IKK activation, revealing the mechanism by which CARMA1 regulates NEMO polyubiquitination should provide further insight about the regulation of the IKK complex.

Negative regulation of CARMA1 and Bcl10 proteins

Posttranslational modifications contribute to the regulation of the expression level of CARMA1. Recent studies indicate that phosphorylation of some residues in CARMA1 may suppress CARMA1 function [70, 103]. The Ser637 residue seems to be phosphorylated by PKC isoforms other than PKCθ or PKCβ, and mutation of Ser637 to Ala enhances CARMA1-induced NF-κB activation [103], suggesting that Ser637 phosphorylation negatively regulates CARMA1 function. Another study

demonstrates that CARMA1 phosphorylation by casein kinase 1α (CK1α) leads to the attenuation of CBM-mediated NF-κB activity [70]. The Ser608 residue within the CARMA1 linker region has been identified as a CK1α phosphorylation site [70], however, the mechanism by which Ser608 phosphorylation suppresses CARMA1 activity remains unclear.

Recent studies indicate that ubiquitination may also regulate the function of CBM proteins. Formation of the CBM complex appears to be negatively regulated by the E3 ligase Cbl-b [73]. It has been shown that Cbl-b mediates monoubiquitination of CARMA1, which disrupts the CARMA1-Bcl10 interaction without affecting CARMA1 protein stability [104]. On the other hand, K48-linked polyubiquitination leads to the proteasome-mediated degradation of CARMA1 [105]. The C-terminal MAGUK region of CARMA1 seems to be involved in this regulation, since SH3 and GUK domains contain the ubiquitin acceptor sites. *In vitro* experiments demonstrate that the cellular inhibitor of apoptosis (cIAP) might be an E3 ligase for CARMA1 [105]. Finally, one study shows that CARMA1 interacts with members of the COP9 signalosome, CNS2 and CNS5 [106]. This work suggests that COP9 regulates IKK activity by maintaining stability of the CBM complex and protecting Bcl10 from degradation [106].

Posttranslational modifications also regulate the level of Bcl10. Although the function of the C-terminal Ser/Thr rich domain of Bcl10 is not fully determined, several studies suggest that signal-dependent phosphorylation of these Ser and Thr residues may mediate degradation of Bcl10 [21, 107-109], thereby terminating NF-κB activation [109]. Indeed, Bcl10-deficient T cells reconstituted with the Bcl10-S138A mutant have prolonged NF-κB activation and enhanced IL-2 production [108]. However, the mechanism by which Bcl10 stability is regulated remains to be defined.

The role of CBM proteins in lymphoma

Previous studies suggest that CBM proteins are involved in lymphoma pathogenesis [30, 31, 41, 110]. Chromosomal translocations, which lead to the overexpression of Bcl10 and MALT1 or generation of API2-MALT1 fusion protein, were found in MALT lymphoma [30, 41, 43], and the activation of NF-κB by these oncogenic proteins is believed to be one of the hallmarks of MALT lymphoma. Consistent with this concept, transgenic mice expressing Eμ-Bcl10 have splenic B-cell expansion and develop marginal zone B-cell lymphoma [111].

Although the CARMA1 gene is not commonly rearranged in B- or T-cell lymphomas, elevated CARMA1 expression was found in adult T cell leukemia [112], primary gastric B cell lymphoma [113] and diffuse large B cell lymphoma (DLBCL) [110]. Importantly, CARMA1 overexpression leads to its oligomerization through the C-C domain and activation of the downstream signaling cascades [114]. Recently, pathogenic oligomerization of CARMA1 with subsequent activation of the CBM complex has been found in the activated B-cell (ABC) subtype of DLBCL [115]. This pathogenic CARMA1 oligomerization results from mutations within exons encoding the C-C domain [115]. Screening of patient samples performed independently by three groups has revealed that CARMA1 is mutated in about 10% of systemic ABC-DLBCL and 16% of primary central nervous system DLBCL [115-117]. The oncogenic mutant of CARMA1 constitutively recruits downstream signaling components [118], and likely induces proteolytic activity of MALT1 [119, 120], leading to activation of NF-κB [115].

Previous studies have shown that NF-κB activity is critical for the survival of malignant cells in ABC-DLBCL [121], and IKK inhibitors [122] or CARMA1 shRNA [110] are toxic for these cells. Therefore, CARMA1 is considered to be an attractive target for the development of specific anti-lymphoma drugs. Although the detailed mechanism of oncogenic function of CARMA1 remains to be fully elucidated, several lines of evidence are consistent with the hypothesis that mutations and/or overexpression of CARMA1 contribute to the lymphoma cell survival. Further studies are needed to determine whether mutation of CARMA1 alone is sufficient to initiate lymphoma and contributes to the malignant phenotype, such as dissemination, of lymphoma.

CARMA3-mediated NF-κB signaling pathways

CARMA3 is expressed widely in non-hematopoietic cells and has recently been described as a link between G protein-coupled receptors (GPCRs) and NF-κB [123]. GPCR is the largest class of transmembrane receptors in the human genome involved in regulation of proliferation, differentiation, and immune response through the wide variety of its ligands [124]. One of them is lysophosphatidic acid (LPA), a bioactive phospholipid that is a component of normal plasma and biological fluids, such as saliva and bronchoalveolar fluid [125, 126]. LPA is capable of inducing diverse cellular responses by inducing activity of several transcription factors, including NF-κB and AP-1 [127].

Genetic deletion of CARMA3 results in diminished LPA-induced NF-κB activation and subsequent IL-8

production in mouse embryonic fibroblasts (MEF) [123]. Similar defect is observed following stimulation with other GPCR ligands, such as endothelin-1 and angiotensin-II, in the absence of CARMA3 [123, 128, 129]. Importantly, CARMA3 is specifically required for GPCR-induced IKK activity because CARMA3 deficiency does not affect IKK activation by other stimuli such as TNFα and lipopolysaccharide (LPS) [123].

Recent study also demonstrates that inhibition of CARMA3 in airway epithelial cells reduces LPA-mediated NF-κB activity and the production of NF-κB-dependent cytokines, TSLP and CCL20 [130]. Both cytokines are produced by airway epithelial cells and play important role in initiating allergic inflammation [131, 132]. Furthermore, forced expression of a dominant-negative CARMA3 mutant (CARD truncation) or treatment of cells with siRNA specifically targeting CARMA3 abrogates LPA-induced signaling in ovarian cancer cells [133].

Similarly to CARMA3, Bcl10- and MALT1-deficient cells have defective NF-κB activation following LPA treatment [134, 135]. These results indicate that the CARMA3-Bcl10-MALT1 complex may play an analogues role in non-hematopoietic cells as the CARMA1-Bcl10-MALT1 complex in the TCR and BCR pathways in hematopoietic cells (Figure 4A). Consistent with this possibility, GPCR-induced ubiquitination of NEMO and activation of the IKK complex is completely defective in the absence of CARMA3 but IKK phosphorylation is intact in these cells [123]. Thus, similarly to CARMA1, CARMA3 mediates the signal-induced polyubiquitination of NEMO (Figure 4B). Interestingly, TRAF6 deficiency also abrogates GPCR-induced NF-κB activation [123]. Therefore, TRAF6 might function as an E3 ligase to induce K63-linked poly-ubiquitination of NEMO leading to activation of the IKK complex. However, further experimental evidence is needed to prove this hypothesis.

GPCR-mediated signaling leads to phosphorylation and activation of different PKC isoforms and pretreatment with PKC inhibitors suppresses GPCR-induced NF-κB activation [6, 123]. To date, it is not clear whether any specific PKC isoform is responsible for phosphorylation of CARMA3. It has been reported that PKCδ mediates NF-κB activation and IL-8 secretion in response to LPA stimulation in bronchial epithelial cells [136]. Moreover, LPA activates PKCα and induces RAS–PKCα interaction, causing NF-κB activation via the CARMA3-BCL10-MALT1 signaling complex in ovarian cancer cells [133]. Therefore, an outstanding question is whether any isoform of PKCs directly phosphorylates CARMA3 and, if it does, which residue of CARMA3 is phosphorylated by PKC. Interestingly, our previous study demonstrates that ectopic expression of CARMA3 in CARMA1-deficient T cells restores TCR-induced NF-κB activation [19], and mutation of Ser520 in CARMA3 (an analogue to Ser552 in CARMA1) to the Ala residue diminished CARMA3's ability to rescue CARMA1 deficiency in T cells [19]. Although Ser520 might be an important phosphorylation site, it is possible that multiple residues in CARMA3 are phosphorylated by PKC, and other kinases may also contribute to CARMA3 activation in the GPCR pathways.

Our previous study has investigated how CARMA3 is linked to the upstream signaling components in the GPCR-induced cascades and found that CARMA3 interacts with β-arrestin-2 [6]. Upon stimulation, GPCR recruits and associates with the multifunctional scaffold molecules, β-arrestins [2]. These proteins were initially considered as components to desensitize GPCR activation but more recent studies indicate that β-arrestins mediate the signal transduction to NF-κB [137]. Although there are four members of the arrestin family in the human genome, only β-arrestin 1 and β-arrestin 2 are ubiquitously expressed in most tissues and function downstream of GPCRs [137]. The study from our laboratory demonstrates that β-arrestin 2, but not β-arrestin 1, is required for LPA-induced NF-κB activation and subsequent IL-6 expression [6]. Mechanistically, β-arrestin 2 associates with CARMA3 and most likely recruits CARMA3 into the receptor complex. Similar to CARMA3-deficient cells, GPCR-induced IKK kinase activity is completely defective in β-arrestin 2 KO mouse embryonic fibroblasts [6].

CBM proteins in the receptor tyrosine kinase pathways

Several growth factors, including Epidermal Growth Factor (EGF) [138-140], Insulin-like Growth Factor (IGF) [141-143], Platelet-Derived Growth Factor (PDGF) [144, 145], and Fibroblast Growth Factor (bFGF) [146, 147], can also induce weak, but notable NF-κB activation through their receptors that belong to a family of receptor tyrosine kinases (RTKs). Although the signaling pathways induced by this family of receptors have been intensively studied, the mechanism by which RTKs activate NF-κB is not fully defined, and the functional significance of RTK-induced NF-κB activation in cell proliferation and survival has not been fully appreciated. Our recent studies indicate that CARMA3 and Bcl10 are required for EGFR-induced NF-κB activation and cancer progression (Jiang and Lin, unpublished data). Therefore, further studies are required to determine how CARMA3 and Bcl10 are involved in the signaling pathways in-

duced by EGFR or other RTKs.

CARD9-mediated NF-κB signaling pathways

CARD9 is another CARD-containing protein and has some similarity to CARMA family members. It was identified through a database search for CARD-containing proteins and shown to interact with Bcl10 [148]. Its expression seems to be restricted to myeloid cells, mainly macrophages, dendritic cells and neutrophils [148-150]. Although CARD9 is structurally related to the CARMA family, it lacks the C-terminal MAGUK domain (Figure 2) that determinates plasma membrane localization. Therefore, CARD9 may localize in cytosol and be recruited to the receptor complex. Recently, three groups independently generated CARD9-deficient mice and revealed its essential role in the control of innate immunity [7, 149, 150].

Initial characterization of CARD9-deficient mice indicates that CARD9 is required for anti-fungal immune responses [149]. This study shows that CARD9 is required for NF-κB activation induced by zymosan, a β-glucan component from yeast cell wall [149]. Because zymosan can activate the signaling pathways induced by Dectin-1, a C-type lectin receptor, it has been proposed that CARD9 mediates Dectin-1-induced NF-κB activation [149]. However, recent studies from our laboratory have surprisingly found that zymosan can still effectively activate NF-κB in macrophages from an independently generated CARD9-deficient mouse strain [151], suggesting that Dectin-1-induced NF-κB activation may also be induced through a CARD9-independent pathway [151]. Therefore, the requirement of CARD9 in Dectin-1-induced NF-κB activation needs to be further investigated. Besides the Dectin-1 pathway, it has been demonstrated that CARD9 also mediates the signaling induced by other C-type lectin receptors such as Dectin-2 [151, 152] and Mincle [153]. These receptors have been implicated to act as the pattern recognition receptors for fungi [154, 155].

It has been suggested that CARD9 functions downstream of the tyrosine kinase Syk and couples Bcl10/MALT1 to activate classical NF-κB [7, 151, 152, 156, 157]. Similarly to CARMA1 and CARMA3, CARD9 is required for activation of the IKK complex [151]. Consistent with this model, CARD9-deficient macrophages and dendritic cells have impaired expression of TNF-α, IL-6, and IL-12 in response to the stimulation with fungal particles [149, 151, 152, 157] and CARD9-deficient mice are more susceptible to infection with the fungus *Candida albicans* [149, 151, 152, 156, 158].

Published studies suggest that Dectin-1 preferentially

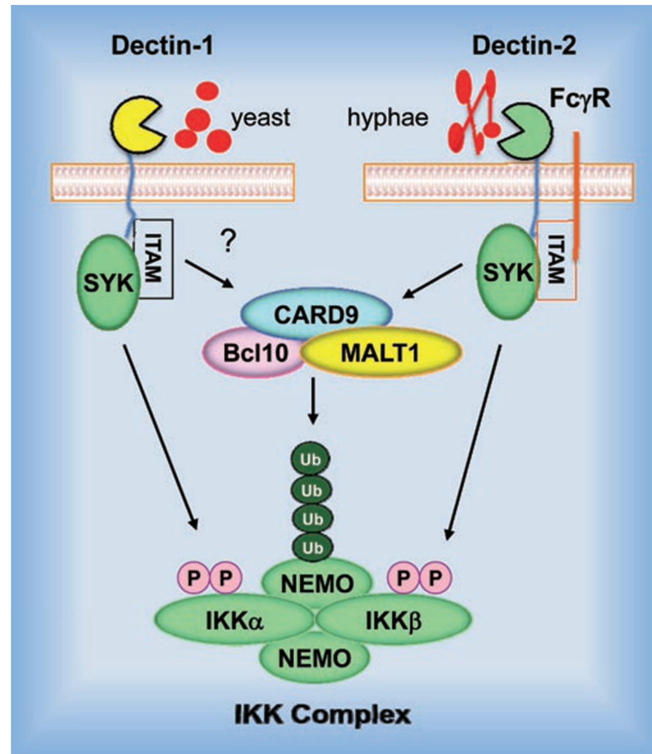


Figure 5 CARD9 in the C-type lectin receptor signaling. Activation of the Dectin-1 receptor by the yeast-like, unicellular form of *C. albicans* or the Dectin-2 receptor by the hyphal form of *C. albicans* leads to a sequential activation of tyrosine kinase Syk, the CARD9-Bcl10-MALT1 complex and IKK. In this signaling cascade Syk mediates IKK phosphorylation, whereas CARD9 controls NEMO polyubiquitination.

binds the yeast-like, unicellular form of *C. albicans*, whereas the Dectin-2 signaling is induced by the hyphal form [152, 158]. The proximal signaling cascade leads to a sequential activation of Syk, CARD9, and IKK following stimulation of these C-type lectin receptors (Figure 5) [13]. However, the latest work argues that although Syk is required for both Dectin-1- and Dectin-2-induced NF-κB activation, CARD9 is only required for Dectin-2-induced NF-κB activation [151]. In addition, Syk and CARD9 do not seem to function in a linear cascade as Syk mediates IKK phosphorylation, whereas CARD9 controls NEMO polyubiquitination [151].

Recent studies also suggest that CARD9 mediates the NF-κB activation induced by several ITAM-associated receptors, including those associating with FcRγ and DAP12 in myeloid cells [7]. Interestingly, it has been proposed that the CARD9-containing complex mediates NF-κB activation induced by the ITAM-associated receptors in myeloid cells, whereas the CARMA1-containing complex mediates NF-κB activation induced by ITAM-

associated receptors in lymphoid lineage cells [8]. However, it remains to be determined why ITAM-associated receptors in myeloid cells utilize the CARD9-dependent, but not CARMA1-dependent, pathway to activate NF-κB, given that CARMA1 is also expressed in myeloid cells.

Other functions of CARD9

Several studies also demonstrate that CARD9 is involved in immunity to intracellular bacteria *Listeria monocytogenes* [7, 150, 159] and *Mycobacteruim tuberculosis* [160]. The latest work suggests that CARD9 controls the production of reactive oxygen species (ROS) by regulating the LyGDI-Rac1 complex following the phagocytosis of microorganisms by macrophages [159]. CARD9 is also found to be involved in anti-viral responses [150, 161]. The CARD9-Bcl10 module appears to be an essential component of the RNA helicase RIG-I-dependent proinflammatory response to certain RNA viruses that leads to IL-1β production [161].

Another study links CARD9 to kidney cancer [162]. It has been shown that tumor suppressor VHL associates with CARD9 and promotes inhibitory phosphorylation of CARD9 by casein kinase 2 (CK2). Inactivation of the *VHL* gene is often observed in renal cell carcinoma and may lead to increased NF-κB activity. Ectopic expression of CARD9 mutants that can not be phosphorylated leads to increased NF-κB activity and decreased apoptosis in VHL-defective renal carcinoma cells, whereas knockdown of CARD9 suppresses NF-κB activity in these cells [162]. Although the role of CARD9 phosphorylation by CK2 is not clear, the authors speculate that this modification may promote an inhibitory intra- or inter-molecular interaction or prevent the binding of CARD9 to NEMO or other proteins required for its activity [162]. One discrepancy of this regulatory mechanism of posttranslational CARD9 modification with previous results is that CARD9 is mainly expressed in myeloid cells and the expression level of CARD9 in renal cells is very low. Nevertheless, future studies are needed to investigate the role of CARD9 in non-myeloid cells.

Concluding remarks

Significant progress towards understanding the function of the CARMA family of scaffold proteins in the NF-κB signaling pathway has been made during the past several years. However, more research is clearly required to define the CARMA-mediated signaling and the role of these proteins in disease, especially in cancer. Still, the open question is the precise molecular mechanism by which CBM activates the IKK complex. Although CARMA/CARD9-dependent NEMO polyubiquitination seems to be important for IKK activity, it is still possible that CBM mediates some additional modifications of the IKK complex members. Moreover, although previous studies indicate that CARMA2 is exclusively expressed in placenta and mucosal cells, the role of CARMA2 is completely unknown. Therefore, further studies are needed to reveal the role of CARMA2 in these tissues.

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