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# Amphioxus SARM Involved in Neural Development May Function as a Suppressor of TLR Signaling

Shaochun Yuan,<sup>1</sup> Kui Wu,<sup>1</sup> Manyi Yang, Liquan Xu, Ling Huang, Huiling Liu, Xin Tao, Shengfeng Huang, and Anlong Xu

Among five Toll/IL-1R resistance adaptors, sterile  $\alpha$  and Toll/IL-1R resistance motif containing protein (SARM) is the only one conserved from *Caenorhabditis elegans* to human. However, its physiologic roles are hardly understood, and its involvement in TLR signaling remains debatable. In this study, we first demonstrated a predominant expression of amphioxus SARM (*Branchiostoma belcheri tsingtauense* SARM) in neural cells during embryogenesis and its predominant expression in the digestive system from larva to adult, suggesting its primitive role in neural development and a potential physiologic role in immunity. We further found that *B. belcheri tsingtauense* SARM was localized in mitochondria and could attenuate the TLR signaling via interacting with amphioxus MyD88 and tumor necrosis receptor associated factor 6. Thus, amphioxus SARM appears unique in that it may play dual functions in neural development and innate immunity by targeting amphioxus TLR signaling. *The Journal of Immunology*, 2010, 184: 6874–6881.

The innate immune system relies on evolutionarily conserved TLRs to recognize diverse microbial molecular structures (1). Most TLRs depend on a family of adaptor proteins containing the Toll/IL-1R resistance (TIR) domain to transduce signals (2). In vertebrates, five TIR adaptors have been identified, including MyD88, MyD88 adaptor-like protein (MAL), TIR-domain containing adaptor inducing IFN- $\beta$  (TRIF), tumor necrosis receptor associated factor 6 (TRAM), and sterile  $\alpha$  and TIR motif containing protein (SARM) (2). MyD88, the first to be described, plays a role in signal transduction by all TLRs except TLR3 (3, 4). When recruited by TLR4 and TLR2, MyD88 reassembles with MAL for the early activation of NF- $\kappa$ B and MAPKs (5–7). TRIF is specifically used by TLR3 and TLR4 to activate NF- $\kappa$ B and another transcription factor, IRF3. TRAM coupled with TRIF is essential for TLR4 signals (6, 8, 9).

The fifth TIR adaptor, SARM, is the only one conserved from *Caenorhabditis elegans* to mammals (10). It is the most recent TIR-containing adaptor protein to be characterized, and little is known about its precise role in immune signaling. A SARM ortholog contains evolutionarily conserved protein structure comprising two sterile  $\alpha$  motifs, an N-terminal heat armadillo repeat motif (ARM), and a C-terminal TIR domain (10). A study of the *C. elegans* SARM homolog (TIR-1) showed its importance to an efficient immune response and in the development of olfactory neurons (11, 12). The knockdown of TIR-1 by RNA interference resulted in decreased *C. elegans* survival when challenged with fungal and bacterial infections (11). However, this effect was independent of TOL-1, the only TLR homolog in *C. elegans* (11).

Unlike other TIR-containing adaptors, overexpression of human SARM in vitro does not induce NF- $\kappa$ B activation. However, it can serve as a negative regulator of TRIF-dependent TLR signaling, but not of MyD88-dependent pathways (13). Overexpression of human SARM leads to the reduction in TRIF-downstream gene expression and enhances TRIF-dependent cytokine production (13). Recently, a SARM ortholog in the horseshoe crab with the ability to downregulate human TRIF-dependent TLR signaling and response to infection was also characterized (14). However, macrophages from SARM knockout mice respond normally to TLR ligands, such as polyinosinic:polycytidylic acid, CpG, and LPS (15). Therefore, the authors concluded that, in contrast to the results of Carty et al. (13), mouse SARM does not have a non-redundant role in regulating TLR signaling. Rather, mouse SARM is primarily expressed in neurons to regulate neuronal death during oxygen and glucose deprivation (15), and its immune function occurs to restrict viral infection and neuronal injury by modulating the activation of resident CNS inflammatory cells (16).

Because the first vertebrate TLR was identified in 1997, an increasingly detailed picture is emerging about its intracellular signaling network. However, no more than 10 studies of SARM have been published, and such limited studies reported different observations among species. Thus, investigating the physiologic roles of SARM in amphioxus, an organism representing a key evolutionary stage from invertebrate to vertebrate, will help to reveal how its role in TLR signaling has been altered, and whether its crucial role in

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Abbreviations used in this paper: ARM, armadillo repeat motif; bbtSARM, *Branchiostoma belcheri tsingtauense* sterile  $\alpha$  and TIR motif containing protein; bbtMyD88, *Branchiostoma belcheri tsingtauense* MyD88; g, gill; HEK, human embryonic; i, intestine; MAL, MyD88 adaptor-like protein; n, notochord; o, ovary; PHB1, prohibitin 1; s, spermary; SARM, sterile  $\alpha$  and TIR motif containing protein; TIR, Toll/IL-1R resistance; TRAF6, tumor necrosis receptor associated factor 6; TRIF, TIR-domain containing adaptor inducing IFN- $\beta$ .

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neuronal development is conserved progressing from *C. elegans* to humans via intermediate species, finally will help to understand the functional transition of TLRs from development to immunity.

## Materials and Methods

### Animals, embryos, cells, and reagents

Adult Chinese amphioxus (*Branchiostoma belcheri tsingtauense*) was obtained from Qingdao, China. During the breeding season, blastulas, gastrulas, neurulas, and larvae were collected. Human embryonic kidney (HEK) 293T cells and Hela cells were maintained in DMEM supplemented with 10% FCS. LPS from *Escherichia coli* (O111:B4), IL-1 $\alpha$  and TNF- $\alpha$  human recombinants were all purchased from Sigma-Aldrich (St. Louis, MO).

### Cloning of *B. belcheri tsingtauense* sterile $\alpha$ and TIR motif-containing protein cDNAs

Our previous annotation of the immune related genes in the *B. floridae* genome identified an SARM ortholog. Based on this sequence, a partial sequence of *B. belcheri tsingtauense* SARM (bbtSARM) was cloned from Chinese amphioxus intestinal cDNA by specific primer pair derived from *B. floridae* SARM. Subsequently, 5'RACE and 3'RACE were conducted according to the manufacturer's protocol using a GeneRACE Kit (Invitrogen, Carlsbad, CA) for full-length sequence cloning.

### Wholemount and section in situ hybridization

A fragment of bbtSARM was amplified and cloned into T-easy vector (Promega, Madison, WI). DIG-labeled sense (synthesized by T7 RNA polymerase) and anti-sense (synthesized by SP6 RNA polymerase) probes were generated based on the T-easy construct according to the manufacturer's protocol using a DIG RNA Labeling Kit (Roche, Mannheim, Germany). Whole-mount in situ hybridizations of embryos of different developmental stages were performed according to the protocol by Holland (17). Section in situ hybridization used the same probes and followed the procedure indicated by Yuan et al. (18).

### Acute immune challenges of adult amphioxus and real-time PCR

Either  $10^5$  CFU of *Vibrio vulnificus* or 15  $\mu$ l per animal of LPS (1 mg/ml) in PBS was injected into the amphioxus coelom. The challenged animals were cultured in separate tanks and collected at 2, 4, 8, 12, and 24 h postinjection. Intestines from five individuals were combined in a single sample for RNA extraction. Intestines from five PBS-injected animals (15  $\mu$ l per animal) were also collected at the same time as controls.

Total RNA from infected samples was prepared, treated with DNAase, and reverse transcribed (Invitrogen). The PCRs were run in triplicate using these conditions: 2 min at 95°C followed by 40 cycles of 30s at 95°C, 15s at 60°C, and 1 min at 72°C. Data were quantified using the  $2^{-\Delta\Delta Ct}$  method based on  $Ct$  values of bbtSARM and  $\beta$ -actin. For expression following challenge, folds were normalized to the expression in PBS injected animals. Values were considered to be significant at  $p < 0.05$ .

### Expression plasmids

For the expression of bbtSARM in 293T cells, PCR fragments encoding for aas 1–734 of bbtSARM fused with 3'Flag tag were inserted into pcDNA3.0. For the coimmunoprecipitation test, the full-length *B. belcheri tsingtauense* MyD88 (bbtMyD88) and *B. belcheri tsingtauense* tumor necrosis receptor associated factor (TRAF6) from Chinese amphioxus fused with flag-tag, and the full length bbtSARM fused with hemagglutinin, were inserted into pcDNA3.0. The vector containing full-length human MyD88 fused with Flag-tag was provided by Dr. H. Tang. For testing the subcellular location of bbtSARM in Hela cells, PCR fragments encoding for aa 1–734, 1–400, 401–734, and 550–734 of bbtSARM were inserted into pEGFP-N1 expression vector (Clontech, Mountain View, CA) and designated as SG-F, SG-1, SG-2, and SG-3.

### Immunofluorescence imaging

Hela cells were seeded onto coverslips (10  $\times$  10 mm) in a 24-well plate for 16 h and transfected with 400 ng indicated expression plasmids. At 24 h posttransfection, cells were fixed for 15 min in a 4% formaldehyde solution, washed twice in PBS, and permeabilized by the treatment for 10 min with PBST (0.1% Triton X-100 in PBS). After blocking for 1 h, cells were incubated with 5  $\mu$ g/ml anti-Flag mAb for 45 min, washed twice in PBST, incubated with a second Ab, triple-washed in PBS, labeled with 0.2  $\mu$ g/ml DAPI in PBS for 5 min, washed for 3–10 min in PBS, and mounted

in MOWIOL R4-88 Reagent (Calbiochem) and photographed by ZEISS AxioVision 4 microscopy (Carl Zeiss MicroImaging, Thornwood, NY).

### Transfection, reporter assays, and measurement of cytokine concentrations

For the basic test, HEK293T cells were plated in 48-well plates ( $2 \times 10^4$  cells per well) and transfected the next day with 400 ng per well DNA. The mixed DNA contained the indicated amount of expression vectors, 10 ng per well of pRL-TK reporter plasmid (Promega) to allow normalization of data for transfection efficiency, and 100 ng per well of the NF- $\kappa$ B response promoter luciferase reporter and the addition of pcDNA3.0. For cytokine stimulation, 100 ng/ml IL-1 $\alpha$  or 100 ng/ml TNF- $\alpha$  were added to the cell medium at 24 h posttransfection as described above, and cells were incubated for an additional 12 h. All samples were measured by a luciferase reporter assay system (Promega) according to the manufacturer's instructions. For measurement of cytokine concentrations, cells were transfected with indicated plasmids, and incubated for 24 h; supernatants were collected, and IL-8 concentration was measured by ELISA (R&D Systems, Minneapolis, MN).

### Coimmunoprecipitation

HEK293T cells in six-well dishes ( $5 \times 10^6$  cells per well) were transfected with 3  $\mu$ g DNA plasmids (1.5  $\mu$ g for each expression vector). At 48 h posttransfection, cells were lysed and incubated with primary Abs (4  $\mu$ g anti-FLAG [M2]; Sigma-Aldrich) at 4°C overnight and then incubated with protein G sepharose for an additional 4 h at 4°C. Analysis was conducted using SDS-PAGE followed by Western blot, using the ECL protocol (Amersham, GE Healthcare, Buckinghamshire, U.K.), with anti-hemagglutinin (1:1000), and anti-Flag (1:1000) mAb.

## Results

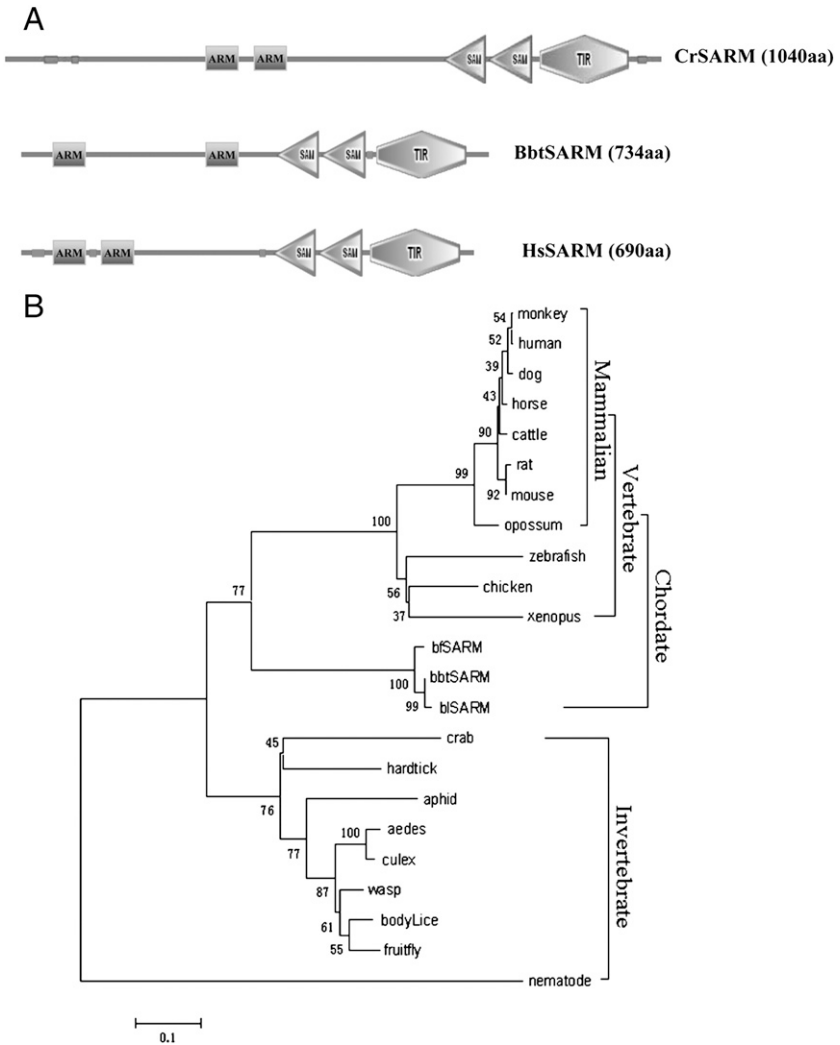
### Cloning and sequence analyses of bbtSARM

A full-length cDNA of 2802 bp was isolated from a *B. belcheri tsingtauense* intestine cDNA library and designated as bbtSARM. With a 600 bp 3'-untranslated region, bbtSARM encoded a polypeptide of 734 aas with a highly conserved protein structure comprising an N-terminal ARM repeat, a C-terminal TIR domain, and two central sterile  $\alpha$  motifs. The overall sequence identity of bbtSARM with other SARM homologs ranged from 33 to 54%, whereas the identity in the TIR domain ranged from 45 to 65%. Compared with horseshoe crab and human SARM homologs (CrSARM and hSARM), bbtSARM was more similar to hSARM, as CrSARM is significantly longer than its vertebrate counterpart (Fig. 1A). Because the N-terminus of CrSARM protein does not contain any recognizable motif, and the N terminal deletion of CrSARM (CrSARM $\Delta$ N) remains functional, the additional N-terminal tail may be redundant and be deleted during evolution (14). A phylogenetic tree showed that the evolutionary position of bbtSARM, which shares a common ancestor with vertebrate SARM, is in accordance with the transition status of amphioxus in the evolution of chordates (Fig. 1B). Thus, the characterization of bbtSARM is of particular importance in shedding light on the evolution and change in the function of SARM from invertebrates to vertebrates and in resolving functional confusion with other reported SARM homologs.

### bbtSARM has dual functions in neuronal development and host defense

Because null-mutant *Drosophila* SARM is lethal and *C. elegans* SARM has a crucial function in the development of olfactory neurons, we used whole-mount in situ hybridization to investigate the potential function of bbtSARM during amphioxus embryogenesis. As shown in Fig. 2, bbtSARM was abundant from zygote to blastula. As embryos developed through the late gastrula, the expression of bbtSARM gradually concentrated in the anterior mesoblast, which develops into the dorsal nerve cord during neurulation. Subsequently, transcripts of bbtSARM were abundant in the mesoblast somite, nerve tube, and notochord from neurula through 24 h larva. In addition, bbtSARM was expressed in the

**FIGURE 1.** Sequence analysis and comparison of bbtSARM. *A*, Domain topology of bbtSARM compared with CrSARM (horseshoe crab) and hsSARM (human). *B*, Neighbor-joining tree of bbtSARM with 23 other SARM sequences was constructed with the MEGA version 3.1 ([www.megasoftware.net/](http://www.megasoftware.net/)) using the full-length sequence. The numbers at the nodes indicate bootstrap values.



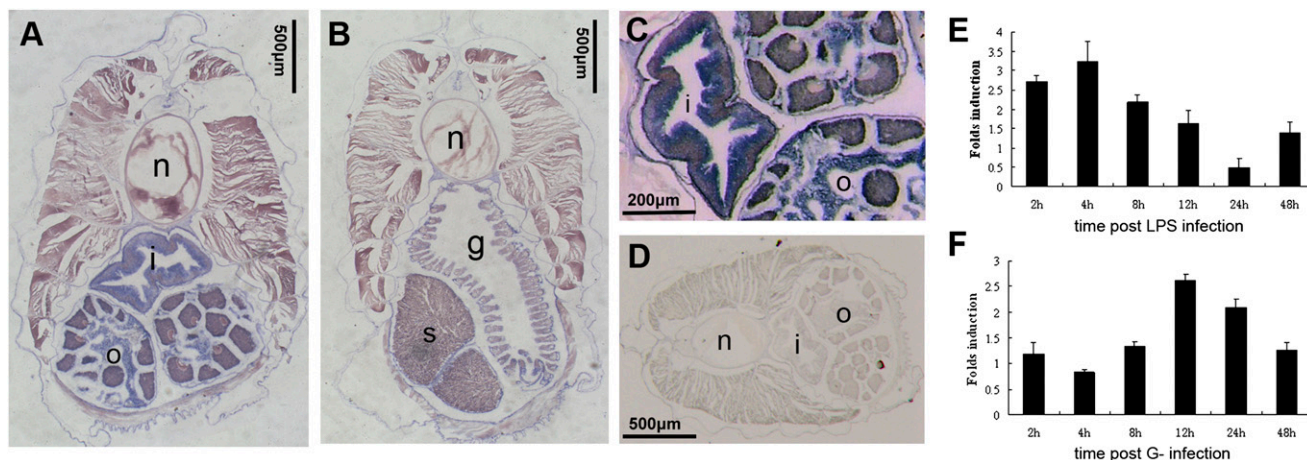
archenteron (the anterior gut diverticulum) during these stages. At 48 h, bbtSARM was detected only in the developing midgut diverticulum, intestine, and atriopore (Fig. 2).

In adult female amphioxus, expression of bbtSARM was strongly detected in the epithelial cells of the gut and weakly in the connective tissue and cell membrane of mature oocytes (Fig. 3A, 3C), but not



**FIGURE 2.** BbtSARM related to neural development. *A*, Four-cell stage. BbtSARM was a maternal expression gene. *B*, BbtSARM was abundant in the blastula. *C* and *D*, Blastopore view and side view of late gastrula showed the expression of BbtSARM in the arterial mesoblast, which develops into the dorsal nerve cord at neurulation. *E–G*, Side view of late neurula, 16-h larva, and 24-h larva, showing that bbtSARM is abundant in mesoblast somites, nerve tube, notochord, and archenteron. *H*, Two-day larva showing bbtSARM only in developing midgut diverticulum, intestine, and atriopore. Scale bar, 100  $\mu$ m for whole embryos. Original magnification  $\times 200$ .





**FIGURE 3.** The tissue distribution and expression pattern after bacterial challenge of bbtSARM. *A*, Section in situ hybridization analyses of bbtSARM anti-sense probe show predominant expression in the intestine, connective tissue, and cell membrane of mature oocytes. *B*, BbtSARM was not abundant on the epidermal cells of the gill, which is also thought to be the defense organ of the amphioxus. *C*, Macroscopic view of the hybridization signals of bbtSARM in intestine and mature oocytes. *D*, Section in situ hybridization analysis of bbtSARM using sense probe as negative control. Scale bar, 500 μm (*A*, *B*, *D*) or 200 μm (*C*). *E* and *F*, Quantitative real-time RT-PCR analysis of the expression of bbtSARM after LPS or Gram-negative bacterial challenge. Results were presented as fold-induction of mRNA expression after PBS injection using the  $2^{-\Delta\Delta C_t}$  method from two parallel experiments done in triplicate. Endogenous control for quantification was cytoplasmic  $\beta$ -actin. Values were considered to be significant at  $p < 0.05$ . g, gill; i, intestine; n, notochord; o, ovary; s, spermary.

abundant in gill epidermal cells and spermatogenic cells (Fig. 3*B*). This predominant expression in the amphioxus digestive system is consistent with that observed in the larva. Because the digestive system is considered to be the primary line of defense in amphioxus, to further confirm the immune significance of bbtSARM, real-time RT-PCR analysis was performed to monitor the expression of bbtSARM transcripts in adults challenged with the Gram-negative bacteria *V. vulnificus* and its cell wall component LPS. In response to acute immune challenge, bbtSARM was not altered dramatically, but was weakly upregulated by Gram-negative bacteria *V. vulnificus* and LPS (Fig. 3*E*, 3*F*). Considering the nature of SARM as an adaptor, this expression profile might imply the immune relevance of bbtSARM in amphioxus. Thus, bbtSARM plays an important role in neuronal development, as demonstrated in other species, and might participate in amphioxus immunity.

#### Amphioxus SARM located in mitochondria

The location of mouse SARM in mitochondria is important for this conserved molecule to recruit JNK3 to regulate neuronal death upon stress (15). To further investigate the functional implications of the subcellular location of bbtSARM, we transfected HeLa cells with a bbtSARM-GFP. Fluorescent imaging microscopy of bbtSARM-GFP fusion protein showed green fluorescence in the cytoplasm at the location similar to that in the mitochondria apparatus (Fig. 4*B*). To investigate whether these punctuated structures of bbtSARM were exactly colocalized with mitochondria, a mitochondria-targeting vector was constructed by fusing a prohibitin 1 (PHB1) sequence into the pEGFP-N1 vector, because PHB1 is an evolutionally conserved mitochondrial protein localized in the inner membrane and has been used as a mitochondria marker in many studies. Next, the PHB1-GFP vector was coexpressed with a bbtSARM-Flag vector that was further costained with rhodamine in HeLa cells (19). As shown in Fig. 4*C*, the bbtSARM-Flag fusion protein colocalized strongly with mitochondria in structures along which mitochondria move. Similar to mouse SARM, the distribution and shape of mitochondria in HeLa cells varied depending on their expression of bbtSARM (Fig. 4*C*). In cells expressing a low to moderate level of bbtSARM, mitochondria were distributed widely throughout the cytoplasm

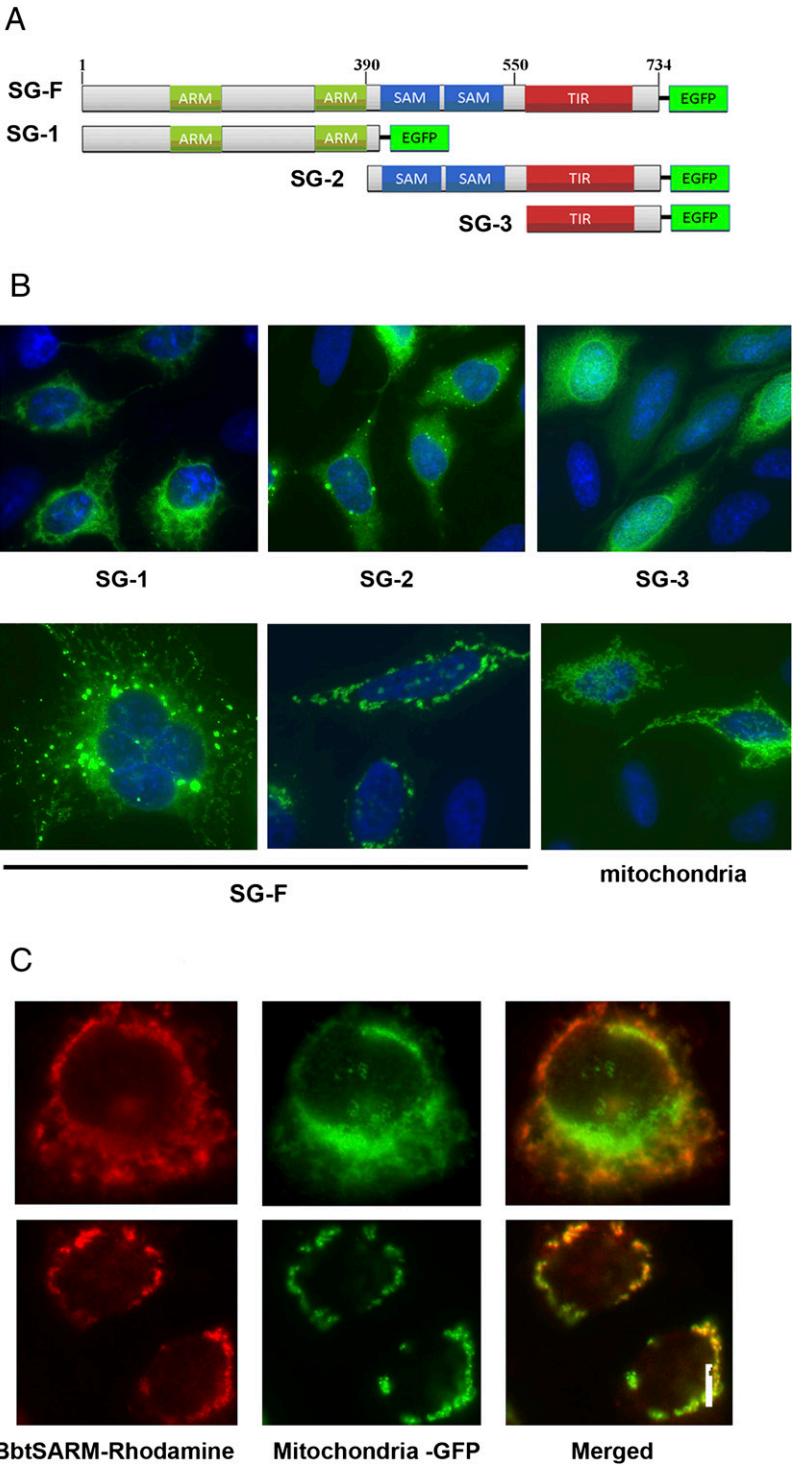
(Fig. 4*C*). In cells with high levels of bbtSARM expression, mitochondria were round and clustered around the nucleus (Fig. 4*C*).

Because we could not predict a mitochondria-targeting sequence in bbtSARM, we used several truncated GFP constructs to verify which domain was crucial for its mitochondrial association (Fig. 4*A*). The truncated segment with only an N terminal ARM repeat showed similar localization, whereas a truncated segment lacking the ARM repeat was instead distributed evenly throughout the cytoplasm (Fig. 4*B*). In contrast, a construct consisting of only the TIR domain was distributed evenly throughout the cytoplasm and the nuclei (Fig. 4*B*). Thus, ARM repeat controls the association of bbtSARM with mitochondria.

#### Amphioxus SARM can attenuate the NF- $\kappa$ B activation mediated by amphioxus MyD88

Given that SARM is a cytosolic TIR domain-containing protein, we sought to determine whether its function is similar to that of other TIR domain-containing proteins. We first compared the ability of bbtSARM and bbtMyD88 to drive transcription factor activation and gene induction. Constant to previous reports, we found that, although overexpression of bbtMyD88 led to activation of NF- $\kappa$ B in 293T cells via a fashion similar human MyD88 (20), overexpression of bbtSARM had no such effect. However, when coexpressed with bbtMyD88, bbtSARM can attenuate the NF- $\kappa$ B activation mediated by bbtMyD88 in a dose-dependent manner (Fig. 5*A*).

To further confirm the observed inhibition on amphioxus MyD88-mediated NF- $\kappa$ B activation, we examined the effect of bbtSARM on human MyD88-dependent gene induction. Results showed that the production of IL-8 (solely mediated via MyD88), was affected by bbtSARM expression in a dose-dependent manner (Fig. 5*B*). In addition, we showed that IL-1 $\alpha$ -induced NF- $\kappa$ B activation, which is mediated exclusively by MyD88, was affected by bbtSARM expression (Fig. 5*C*). Because the activation of NF- $\kappa$ B is also a hallmark of TNF signaling, to determine whether the inhibition by bbtSARM is restricted to TLR signaling, we investigated the effect of bbtSARM expression on NF- $\kappa$ B activation induced by TNF- $\alpha$ . Results showed that overexpression of bbtSARM did not affect the TNF- $\alpha$ -induced NF- $\kappa$ B activation (Fig. 5*C*), suggesting that bbtSARM may be a specific suppressor for the TLR signaling.

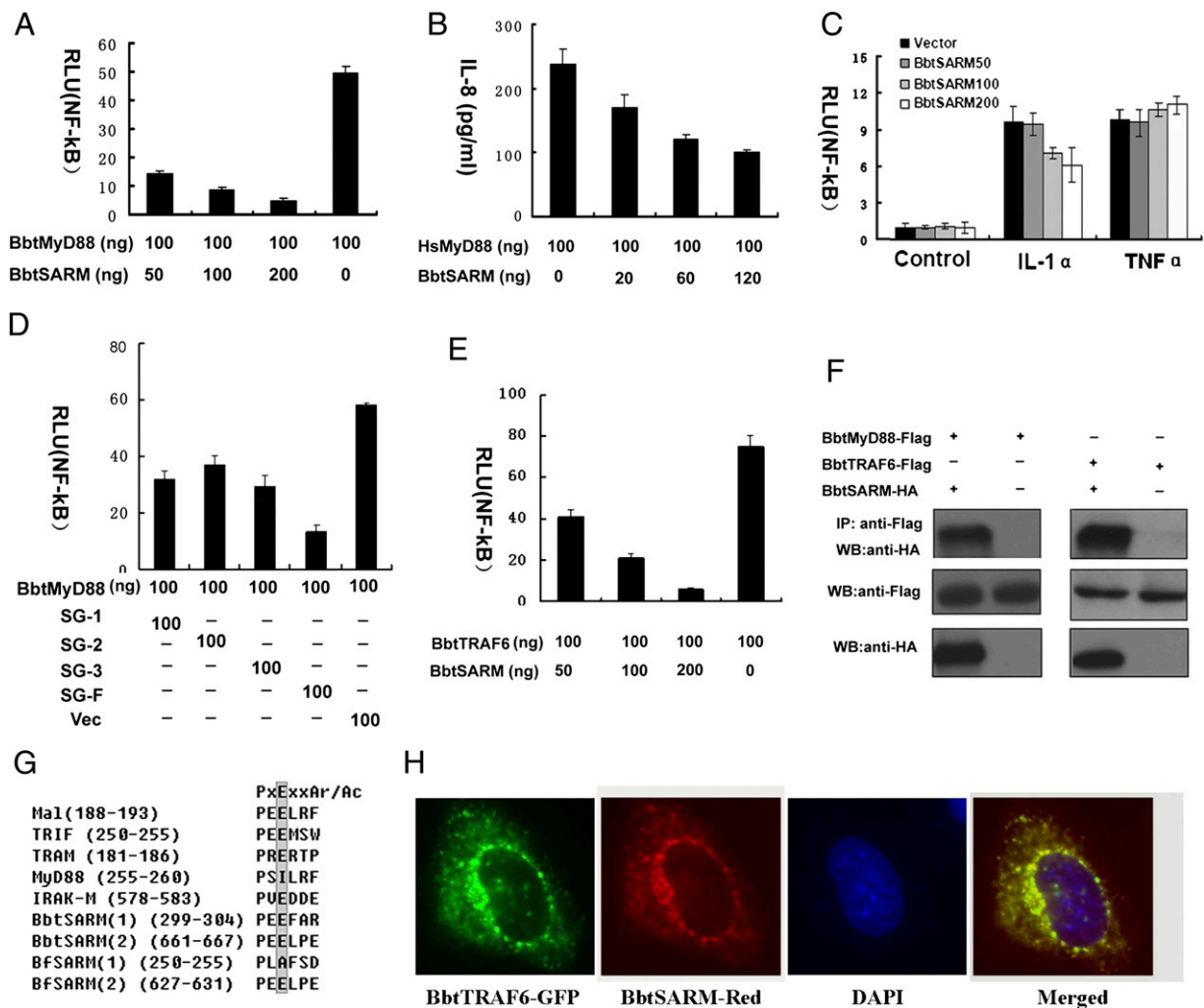


**FIGURE 4.** Mitochondria localization of bbtSARM. *A*, Name and structure of fusion proteins used in this study. *B*, Intracellular localization of bbtSARM and its truncated mutants fused with EGFP in HeLa cells. *C*, bbtSARM was well colocalized with mitochondria, and the shape of mitochondria in HeLa cells varied depending on their expression of bbtSARM. Nucleus was stained by DAPI. Original magnification  $\times 400$  (*B*),  $\times 630$  (*C*).

*Amphioxus SARM may target MyD88 and TRAF6*

To obtain further information on the mechanism of bbtSARM function in TLR signaling, we transfected the several truncated mutants with amphioxus MyD88 for luciferase activity analyses. As shown in Fig. 5*D*, all truncated mutants are functional. Given the presence of the TIR homotypic interaction domain in those TIR domain-containing adaptors, we speculated that SARM inhibition of MyD88 signaling might be accomplished through its direct interaction with amphioxus MyD88. To test that possibility, we conducted coimmunoprecipitation trials. Results showed bbtSARM to interact with bbtMyD88 (Fig. 5*F*). We further observed that overexpression of bbtSARM resulted in the inhibition of NF- $\kappa$ B activation mediated

by amphioxus TRAF6 (Fig. 5*E*), which shares high sequence conservation and functions in a manner similar to human TRAF6 (18). Thus, sequence analysis was performed to discover whether bbtSARM contained a PxExxAr/Ac TRAF6-binding motif, and then two putative TRAF6-binding motifs were identified—one at aa position 299–304 and a second at 661–667 (Fig. 5*G*). Such motifs have not been identified in other SARM homologs, but have been described in IL-1R-associated kinases, MAL, and TRIF (21, 22). To test whether bbtSARM can interact with bbtTRAF6, coimmunoprecipitation experiments were performed and demonstrated that bbtSARM interacted with bbtTRAF6 (Fig. 5*F*). We also observed that bbtSARM can colocalize with bbtTRAF6 in HeLa cells (Fig. 5*H*).



**FIGURE 5.** Analysis of bbtSARM activity on TLR signaling. *A*, bbtSARM can attenuate the NF-κB activation mediated by amphioxus MyD88. *B*, Overexpression of bbtSARM inhibited the IL-8 secretion mediated by human MyD88. *C*, Activation of NF-κB through 100 ng/ml IL-1α, but not 100 ng/ml TNF-α, was inhibited by bbtSARM in a dose-dependent fashion. *D*, All three domains are crucial for bbtSARM to perform negative regulatory activities in the MyD88-dependent pathway. *E*, bbtSARM inhibited the NF-κB activity induced by amphioxus TRAF6 by reporter assays. *F*, bbtSARM interacts with amphioxus MyD88 and TRAF6 when overexpression in 293T cells. *G*, Putative TRAF6-binding motifs found in amphioxus SARM. *H*, BbtSARM is colocalized with amphioxus TRAF6 in Hela cells (original magnification ×630). All experiments were done in triplicate and repeated at least twice in all cases. Data were expressed as fold-induction (mean ± SD) relative to control induction for a representative experiment.

These data suggest that TRAF6 may be another target for bbtSARM to serve as a suppressor of TLR signaling.

**Discussion**

*Shift of SARM function between development and innate immunity*

The SARM ortholog in *C. elegans*, TIR-1, participates in the development of olfactory neurons by assembling a synaptic signaling complex that regulates odor receptor expression (23). The greater expression of mouse SARM in the brain suggested that its function is mainly neuronal, and the study of SARM-deficient mice revealed its role in the regulation of neuronal survival in response to metabolic stress (15, 24). Based on our observation, bbtSARM was maternally expressed, predominately in neuron-related cells from gastrula to 24-h larva, suggesting a conserved role of SARM in neuronal development among species, including in the basal chordate amphioxus. Similar to mouse SARM, which is localized in part of the mitochondria (15), bbtSARM is largely colocalized with mitochondria, in structures along which mito-

chondria move. Because of the lack of amphioxus cell lines at present, it still needs to be established whether bbtSARM participates in neural development as mouse SARM by a trigger JNK pathway to control apoptosis.

*C. elegans* TIR-1 also played a role in immune defense. Inactivation of TIR-1 by RNA interference was associated with increased susceptibility to infection (11, 25). Our expression analysis indicated that bbtSARM was concentrated in intestine, diverticulum, and anus from 48-h larva to adult amphioxus. In addition, bbtSARM was upregulated after LPS and Gram-negative bacteria challenge. SARM in the horseshoe crab was also upregulated by infection (14). These studies suggested that SARM also can function as a component of host defense in invertebrates. The immune function of mouse SARM is showed to restrict viral infection and neuronal injury by modulating the activation of resident CNS inflammatory cells (16). Thus, further studies of bbtSARM during amphioxus embryogenesis might reveal similarities in the way neurons and immune cells sense and respond to danger, which might help to elucidate the cross talk between neuronal cells and immune cells during evolution.



### Potential function of SARM in innate immunity by targeting TLR signaling

Among five TIR adaptors, SARM is the only one conserved from *C. elegans* to mammals. The prominent contribution of all other known TIR adaptors to innate immunity is well documented and has fueled the expectation that SARM would play a similar role. However, although *C. elegans* TIR-1 has a positive function in immunity, it does not appear to mediate signaling from *C. elegans* TOL, the sole TLR in *C. elegans*, but acts as a component of a p38 MAPK signaling cassette (11, 12, 26). In contrast, human SARM is a specific negative regulator of TRIF signaling through its targeting of TRIF for innate immune responses (13). Subsequent to the study by Carty et al. (13) a study of mouse SARM showed that it does not have a nonredundant role in regulating macrophage responses to polyinosinic:polycytidylic acid and LPS, which may rule out a role in TLR signaling (15). Thus, the involvement of SARM in TLR signaling is still debatable, and the molecular basis for this functional difference remains unclear.

Because the amphioxus genome does not contain an ortholog to mammalian TRIF, IRF3, IRF7, and IFN- $\beta$ , the MyD88-dependent pathway seems to be the key route of TLR signaling in the amphioxus (27). In addition, amphioxus MyD88 and TRAF6 share high sequence similarities and function in a fashion similar to their human counterparts in HEK293T cells, indicating the molecular conservation of the MyD88-dependent pathway between amphioxus and humans (18, 20). Therefore, our observation that bbtSARM could attenuate the NF- $\kappa$ B activation mediated by amphioxus MyD88 and TRAF6 in 293T cells may present some natural roles of this conserved molecule in amphioxus cells, adding evidence that SARM may be a negative regulator of TLR signaling at the basal chordate stage. Considering its roles in neural development, further study to reveal how bbtSARM regulates neural development by targeting MyD88 dependent pathway would shed light on the alternation of TLR function between development and immunity when invertebrates developed into vertebrates.

### Molecular basis for bbtSARM to participate in TLR signaling

Although the ARM repeat for mouse and amphioxus SARM is important for their localization with mitochondria (15), this specific localization seems not to be indispensable for bbtSARM in TLR signaling, as the truncated mutant without ARM repeat is still functional. The *C. elegans* TIR-1, when truncated to only the two sterile  $\alpha$  motifs and the TIR domain, showed a stronger gain-of-function developmental phenotype than the full length protein (11, 25). The N terminus-deleted SARM in humans and horseshoe crab were also more efficient in the inhibition of TRIF-dependent pathways (13, 14). These data suggest that the negative roles of SARM in TLR signaling may be determined by its sterile  $\alpha$  motifs and TIR domain, which provide interface to interact with different molecules. Similar to other TIR adaptors, the TIR domain of bbtSARM mediated the direct TIR-TIR interactions. For example, amphioxus SARM could interact with MyD88, whereas human SARM interacts with TRIF. It may be that the binding of SARM to MyD88 prevents the formation of the MyD88 complex (28). Unlike the TIR domain, proteins with sterile  $\alpha$  motifs exist in all subcellular locations and could form multimeric complexes with a wide variety of proteins (29), providing another interface for bbtSARM to interact with some unidentified molecules involved in TLR signaling. In addition, we identified two TRAF6-binding motifs in bbtSARM protein and confirmed their direct interaction when overexpression. Thus, another possibility may be that the interaction of SARM with TRAF6 physically prevents engagement of TRAF6 with its upstream activators or downstream

effectors (30, 31). Therefore, we are not surprised to observe that all three conserved protein domains found in bbtSARM are necessary for its inhibition activity in TLR signaling. Owing to the lack of amphioxus cell lines for functional analyses, we have to borrow a mammalian cell line system to study the functions of amphioxus molecules, which may present limitations for these cross-species approaches. Nevertheless, this first report of amphioxus SARM establishes its conserved roles in neural development and provides evidence for its involvement in innate immunity, which should help to provide a picture on the functional evolution of SARM from *C. elegans* to humans.

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### Disclosures

The authors have no financial conflicts of interest.

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