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Deleted in azoospermia-associated protein 2 regulates innate immunity by stimulating Hippo signaling in crab

Running title: Protein control of crab immunity via the Hippo pathway

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Abstract

The Hippo signaling pathway plays a critical role in both normal animal physiology and pathogenesis. Since pharmacological interventions targeting this pathway have diverse clinical implications, a better understanding of its regulation in various conditions and organisms is crucial. Here, we identified deleted in azoospermia-associated protein 2 (DAZAP2) in Chinese mitten crab (*Eriocheir sinensis*), designated *Es*DAZAP2, as a Hippo-regulatory protein highly similar to proteins in various species of insects, fish, and mammals. We found that a bacterial infection significantly induces *Es*DAZAP2 expression and an *Es*DAZAP2 knockdown both suppresses antimicrobial peptide (AMP) expression *in vitro*, and results in increased viable bacterial counts and mortality *in vivo*, suggesting that *Es*DAZAP2 plays a critical role in innate immunity. Using yeast two-hybrid screening and co-immunoprecipitation assays, we found that *Es*DAZAP2 regulates the Toll pathway rather than the immune deficiency (Imd) and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways. Our findings also demonstrate that *Es*DAZAP2 binds to the Hippo protein, Salvador (Sav). Moreover, by examining the regulation of Dorsal, a trasnscription factor that regulates AMP expression in *E. sinensis*, we provide experimental evidence indicating that *Es*DAZAP2 promotes Hippo pathway activation in innate immunity, with *Es*DAZAP2 and Hippo binding to different Sav domains. To the best of our knowledge, this is the first report of a DAZAP2-regulated Hippo signaling pathway operating in animal innate immunity.

Introduction

Invertebrates, such as insects and crustaceans, rely solely on innate immunity for their immunological defense, and so have evolved complex mechanisms to counter microbial

infections (1). Pioneering studies in *Drosophila* delineated two key signaling pathways, namely Toll and Imd, responsible for the immune response that functions through the production of antimicrobial peptides (AMPs) (2,3). The Toll pathway mainly detects fungi, Gram-positive bacteria, and virulence factors (e.g., proteases). Once this pathway is activated, degradation of Cactus ensues with Dorsal and Dif localized to the nucleus (2). By contrast, the Imd pathway responds to Gram-negative bacteria and leads to the subsequent activation of Relish, which along with Dorsal and Dif induce the transcriptional up-regulation of AMPs in the nucleus (3). The field of immunology is rapidly advancing, largely due to its incorporation of molecular and cellular biological techniques; this lets us identify novel immune signaling pathways and their regulators, which are needed to illuminate whole immune networks and their functioning.

The Hippo signaling pathway is a central regulator of organ size in a wide variety of animals, from *Drosophila* to mammals (4,5). In *Drosophila*, the core of the Hippo pathway has four proteins: Hippo (Hpo), Salvador (Sav), Warts (Wts), and Mob-as-tumor-suppressor (Mats), which together form the Hippo kinase cassette (6). Loss-of-function mutations in any of these four genes will result in excess growth due to increased cell proliferation coupled to reduced cell deaths (7). The activation of this signaling axis will exclude Yki from the nucleus, where it normally functions as a transcriptional co-activator for the expression of target genes (8-10). Recent work from multiple model systems now suggest that the Hippo kinase cascade represents a signaling module that integrates multiple biological inputs, accentuating its importance in organismal growth control (11,12). Not surprisingly, dysregulation of Hippo signaling has been linked to a variety of human cancers (13,14).

Most studies to date on Hippo signaling have focused on its role in developing, or regenerating tissues of animals. Although that body of work has firmly established a critical role for

Hippo signaling in tissue growth, differentiation, regeneration, and homeostasis, whether and how the Hippo pathway might participate in non-developmental and non-growth-related processes remains largely unexplored. Innate immunity is the nonspecific, first line of defense against foreign pathogens; interestingly, Hippo pathway-regulated innate immunity has been detected (6,15,16), expanding our understanding of its potential. Specifically, canonical Hippo-Yki signaling was found acutely activated by Gram-positive bacteria, with the antimicrobial response then enhanced via lowered expression of Cactus (the IkB homolog), an Yki target gene (6). In mouse macrophages, infection by *Mycobacterium tuberculosis* also activated Mst1/2 phosphorylation in a TLR2-IRAK1/4-dependent manner, but independently of the canonical Hippo pathway (15). Furthermore, Mst1/2 in myeloid cells can promote the direct killing of phagocytosed bacteria, by inducing the juxtaposition of phagosomes with reactive oxygen species (ROS)-producing mitochondria through Rac activation (16). Taken together, these above studies reveal critical roles of the Hippo pathway in antibacterial immunity through several different mechanisms. Nevertheless, how these mechanisms are coordinated in different environments and their regulators' involvement still awaits determination.

The DAZ-associated Protein 2 (DAZAP2) gene encodes a small protein that is highly conserved throughout evolution, with the most notable features being a high proline content and several potential Src homology 2 (SH2)- and SH3-binding motifs (17). DAZAP2 mRNA and protein expression are frequently down-regulated in multiple myeloma patients whilst DAZAP2 mRNA has been shown to be increased in adhering mouse osteoblasts or rat astrocytes grown in high ammonia or hypo-osmotic conditions (18). The interaction between DAZAP2 and eukaryotic initiation factor 4G (eIF4G) is essential for the formation of discrete cytoplasmic foci (19). Moreover, the

level of DAZAP2 protein expression is regulated by its interaction with neural precursor cells expressing developmentally downregulated 4 (NEDD 4) (20). Moreover, a knockdown of DAZAP2 could both reduce the level of Wnt-signaling activity and additionally alter the expression of its target genes (21). Taken together, the aforementioned data indicate that DAZAP2 functions in diverse roles that include cell signaling, transcription regulation, and pathogenesis of multiple myeloma in cell biology and physiology. However, the immune functions and potential signal regulation activities of DAZAP2 remains largely unknown in invertebrates.

Chinese mitten crab, *Eriocheir sinensis*, is one of the most important freshwater aquaculture economic products in China; however, high-density culture leads to a burst of bacterial disease in different culture locations (22). Hence, exploring the anti-bacterial immune reactions of crab will both provide a theoretical basis for disease control, as well as a better understanding of the innate immune system of invertebrates, which lack adaptive immunity. Here, we report on the identification and characterization of the DAZ-associated Protein 2 (DAZAP2) protein as an essential regulator of Hippo pathway in the Chinese mitten crab, *Eriocheir sinensis*. We show that DAZAP2 participates crucially in anti-bacterial immune reactions by regulating dorsal-controlled AMPs' expression. We further show DAZAP2 protein binding with SAV, which regulated Hippo signaling activation and the translocation of Yki from the cytoplasm into nucleus. We also demonstrate Hippo signaling in crab is capable of regulating antibacterial immunity using similar signaling transduction processes as in *Drosophila* (6). Furthermore, we show DAZAP2 and Hpo bind to different domains in Sav, which cooperatively may promote Hippo signaling activation. Our study's complimentary experiments have elucidated a novel Hippo signaling regulator of innate immunity and thus

implicate Hippo signaling as being tightly regulated during a bacterial infection.

Results

cDNA cloning and expression pattern of DAZAP2

The full-length DAZAP2 cDNA was cloned from hemocytes of *Eriocheir sinensis*. The DAZAP2 gene contained a short 5' untranslated region (UTR), a relatively long 3' UTR, plus a poly(A) tail and an ORF. The ORF is predicted to encode a 26.7-kD protein comprising 252 amino acids. The DAZAP2 proteins contain a proline-rich region at their C-terminus and several potential SH2 (Yxxψ) and SH3 (PxψP) domain-binding motifs (Fig. 1A and B). Our phylogenetic analysis revealed two major branches that included vertebrates and invertebrates, with *Es*DAZAP2 clustered within the latter and close to insects (Fig. 1C), consistent with classical zoological systematics. The DAZAP2 gene was expressed in different tissues harboring hemocytes (Fig. 1D), which hinted at its role in crab physiology. To test whether DAZAP2 expression could be induced post infection, *S. aureus* (Fig. 1E) and *V. parahemolyticus* (Fig. 1F) were used to infect crabs *in vivo*. Soon after infection with either pathogen, this significantly induced the expression of DAZAP2, thus demonstrating its potential participation in innate immunity.

DAZAP2 controlled the antibacterial activities in hemocytes

To study the role of DAZAP2 in innate immunity, siRNA was injected into crabs *in vivo*, and the expression of DAZAP2 in their hemocytes showed an effective inhibition (Fig. 2A). This was followed by greatly reduced crab survivorship and significantly enhanced bacterial concentrations in the hemolymph since infection with *S. aureus* (Fig. 2B, D) or -*V. parahemolyticus* (Fig. 2C, E). The vital role of AMPs in antibacterial immune reactions of invertebrates is certain, and some AMPs did show pathogen-specific induction after the bacterial infection. We then examined the expression patterns of AMPs in bacteria-stimulated hemocytes, finding that some AMPs

were significantly induced; those including ALF1, ALF2, Lys, and DWD1 were selected for further investigation because they were strongly induced by both *S. aureus* (Fig. 2F) and *V. parahemolyticus* (Fig. 2G) infections. This result supports the prior one that suggested DAZAP2 could regulate the innate immunity of crabs infected by *S. aureus* or *V. parahemolyticus.* To test whether DAZAP2 regulated AMPs' expression, it knocked down in hemocytes *in vitro*: this significantly inhibited AMPs expression after stimulation with either *S. aureus* (Fig. 2H) or *V. parahemolyticus* (Fig. 2I). Together, these results demonstrated a critical involvement of DAZAP2 in antibacterial immune reactions.

DAZAP2 regulated AMPs expression via dorsal

The importance of Toll, IMD, and JAK/STAT signaling pathways in AMPs' expression has been widely confirmed in invertebrates (2,3,23). To test whether DAZAP2 could regulate AMPs expression via these pathways, immunocytochemical staining assays were used to determine the translocation of Dorsal, Relish, and Stat92E from cytoplasm into nuclear in DAZAP2- or GFP-silenced hemocytes after *S. aureus* or *V. parahemolyticus* stimulation. Results clearly show that Dorsal (Fig. 3A left), Relish (Fig. 3A middle), and Stat92E (Fig. 3A right) were translocated post-bacterial stimulation (both species); however, DAZAP2 only regulated the translocation of Dorsal from the cytoplasm into nucleus, which demonstrated it may regulate AMPs' expression via the Toll pathway. Going one step further, we effectively knocked down Dorsal expression in hemocytes (Fig. 3B), which revealed the expression levels of AMPs regulated by DAZAP2 were significantly reduced in Dorsal-silenced hemocytes after infection with *S. aureus* (Fig. 3C) or *V. parahemolyticus* (Fig. 3D).

DAZAP2 interacted with Sav

Considering the plausible role of Dorsal in DAZAP2-regulated AMPs' expression, we speculated that DAZAP2 may bind with molecules or regulators in the Toll pathway, to regulate Dorsal

translocation and subsequent AMP expression. To identify such DAZAP2-interacting proteins, and to elucidate the underlying molecular mechanism for AMPs' regulation, an Y2H screen was performed (Fig. S1). The ORF of DAZAP2 was successfully cloned in frame with the GAL4DNA-binding domain of the pGBKT7 vector (Fig. 4A), with the ensuing construct transformed into an yeast Y2H Gold strain. The pGBKT7-DAZAP2 bait protein did not autonomously activate the reporter genes and was confirmed as nontoxic (Fig. 4B). Unexpectedly, an initial screen identified Sav—a core component of the Hippo signaling pathway—as predominantly interacting with DAZAP2 (i.e., 12 of 26 positive clones). To distinguish genuine positive from false positive interactions, the pGADT7-Sav prey plasmid had to rescued from the yeast, for auto-activation testing. These results showed the pGADT7-Sav prey protein failed to activate the reporters (Fig. 4C). Further evidence confirming the physical interaction between DAZAP2 and Sav came from both the Y2H (Fig. 4D) and *in vivo* co-immunoprecipitation assays in crab hemocytes (Fig. 4E).

DAZAP2 regulated Hippo signaling activation

Due to DAZAP2 being able to interact with Sav, an important molecule in Hippo signaling, we wondered whether DAZAP2 regulated the Hippo pathway's activation in crab. Hpo, Sav, and Yki were conserved among different species (Fig. S2). In *Drosophila*, it is known that Hpo becomes phosphorylated after binding with Sav, which inhibits the Yki translocation from cytoplasm into nucleus. To test whether the same process operated in crab after an immune challenge, hemocytes were stimulated by *S. aureus* or *V. parahemolyticus*, which clearly increased phosphorylation of Hpo at 0.5 h and 1 h post-stimulation (Fig. 5A). Moreover, Yki did not reach the nucleus in hemocytes, after either *S. aureus* or *V. parahemolyticus* stimulation at 0.5 h and 1 h (Fig. 5B). Given the key importance of Hpo and Yki in Hippo signaling, we also analyzed the phosphorylation rate

of Hpo in DAZAP2-silenced hemocytes after bacterial stimulation. This revealed that DAZAP2 was able to regulate Hpo phosphorylation at 1 h after stimulation by either *S. aureus* or *V. parahemolyticus* (Fig. 5C). Moreover, most Yki were translocated from the cytoplasm into nucleus in DASZAP2-silenced hemocytes following the *S. aureus* or *V. parahemolyticus* stimulation (Fig. 5D). Collectively, these results demonstrated the pivotal role played by DAZAP2 in Hippo signaling regulation.

Hippo signaling regulated AMPs' expression in hemocytes

The essential role of Hippo signaling in antibacterial activities has only been reported in *Drosophila* (6). Hpo is also required for an antimicrobial response in *E. sinensis* (Fig. S3). To test whether Hippo signaling also influenced AMPs expression in crab, Hpo was knocked down in hemocytes (Fig. 6A), which clearly showed decreased Dorsal in the nucleus after the *S. aureus* and *V. parahemolyticus* stimulations (Fig. 6C). Since dorsal translocation is inhibited by Yki-regulated Cactus expression in *Drosophila*, we effectively knocked down Yki expression in hemocytes (Fig. 6B) and this significantly decreased the expression of Cactus after stimulations with *S. aureus* and *V. parahemolyticus* (Fig. 6D). To study whether DAZAP2 regulates Cactus expression, DAZAP2 was knocked down in crab hemocytes, which led to a significant upregulation of Cactus transcription following stimulation with *S. aureus* and *V. parahemolyticus* (Fig. 6E). Furthermore, those AMPs regulated by DAZAP2 had very significantly suppressed expression levels in Hpo-silenced hemocytes, after the *S. aureus* (Fig. 6F) and *V. parahemolyticus* (Fig. 6G) stimulations. These results demonstrated that Hippo signaling can also regulate AMPs' expression in crab.

DAZAP2 and Hpo interacted with different domains of Sav

The protein complex of Sav and Hpo are essential to carry out Hippo signaling transduction (5,6). We found that DAZAP2 can also interact with Sav, but we still did not know whether DAZAP2

and Hpo interacted with same or unique domain of Sav. For this purpose, plasmids containing the full ORF regions of Hpo, DAZAP2, and Sav, and a truncated ORF region of Sav lacking the SARAH or WW domain were constructed (Fig. 7A). After transfecting these plasmids into *Drosophila* S2 cells, Co-IP assays revealed all of them were well expressed, and that DAZAP interacted with the full-length SAV via the WW domain but not the SARAH domain (Fig. 7B). Conversely, Hpo interacted with the full-length Sav via the SARAH domain but not the WW domain (Fig. 7C). To study the relationship between Hpo, Sav, and DAZAP2, a Co-IP assay was conducted in crab hemocytes, and the results demonstrated the interaction between these proteins *in vivo* (Fig. 7D). Thus, these results demonstrated that Hpo, Sav, and DAZAP2 can form a tri-molecular complex by the interaction of the WW and SARAH domains in Sav with DAZAP2 and Hpo, respectively.

Discussion

An emerging theme of biological systems is that a limited number of signaling pathways are used reiteratively to control myriad biological processes. In invertebrate immunology research, a few such signaling pathways have been clearly demonstrated and widely confirmed in different species, mainly those of Toll (2), IMD (3), and JAK/STAT (23). Recently, however, an essential role for Hippo signaling, a developmental signaling pathway, in innate immune response was reported (6). Toll receptor and Hippo signaling are implicated in other biological processes, such as cell competition in the wing disc (24,25) and polarized cell rearrangements in *Drosophila* embryos (26,27). That research in *Drosophila* emphasizes these seemingly disparate processes (Hippo and Toll) are functionally intertwined suggests their deep evolutionary origin. Our findings here of the Hippo signaling pathway's key role in *E. sinensis* crabs expands the current knowledge of crustacean innate immunity, and DAZAP2 binding with Sav should help advance our

understanding of the Hippo pathway's regulation and function in animals.

DAZAP2 has a highly conserved sequence throughout evolutionary history, including a conserved polyproline region and several SH2/SH3 binding sites. Its gene encodes a ubiquitously expressed protein and binds to DAZ and DAZL1 through DAZ repeats. Yet DAZAP2 has been studied little to date. What is known is that DAZAP2 was originally identified as an interacting protein of germ cell-specific RNA-binding proteins deleted in azoospermia (DAZ) (28); it was found associated with pathogenesis of multiple myeloma (MM) (18); and its degradation is critical for IL-25 signaling and allergic airway inflammation (29). In our study, the expression of *Es*DAZAP2 was significantly induced post-bacterial infection and *Es*DAZAP2 positively regulated the AMPs' expression, bacterial clearance, and host crab survivorship, which spurred us to explore the immune mechanisms of *Es*DAZAP2. Due to the crucial functioning of Toll, IMD, and JAK/STAT pathways in invertebrate innate immunity (2,3,23), we tested the respective translocation of Dorsal, Relish, and Stat92E in *Es*DAZAP2-silenced hemocytes, finding that only dorsal could be regulated, thus indicating a possible relationship between *Es*DAZAP2 and the Toll pathway. However, Y2H screening using *Es*DAZAP2 as bait captured Sav, which is a signaling molecular in Hippo pathway, an association confirmed by our Co-IP assay. It is thus clear that the SARAH domain in Sav binds with the same domain in Hpo to promote the latter's phosphorylation. Our results also demonstrated that the DAZAP2 domain in *Es*DAZAP2 binds with the WW domain in Sav, which suggests DAZAP and Hpo bind different regions of Sav and that this protein trimer affects the phosphorylation of Hpo and the nuclear translocation of Yki.

Recent studies have revealed a new paradigm of Hippo signaling in the immune system of animals (6,30,31). These findings remind us that the Hippo

pathway is not exclusively a developmental pathway, being capable of specific functions in differentiated tissues. Of particular interest to us is Gram-positive bacteria rather than Gram-negative bacteria activating the *Drosophila* Hippo pathway: inhibition of the Hippo pathway suppressed only Gram-positive bacteria-induced innate immunity, as this prevented Yki translocation, leaving Cactus unable to bind with a transcription factor to promote its transcription, and this low expression of Cactus would have released Dorsal/Dif from the cytoplasm into nucleus, thereby enhancing AMPs' expression (6). Our study thus demonstrated the vital contribution of the Hippo pathway and its crosstalk with the Toll pathway to the innate immunity of the *E. sinensis* crab, which suggests such an immune-related function for the Hippo pathway may also exist in other invertebrate species. Interestingly, both Gram-positive and Gram-negative bacteria induced the activation of Hippo pathway in this crab, and its Hpo-silenced hemocytes showed significantly low expression of AMPs. In insects, the Toll pathway responds to Gram-positive bacteria (2) whereas the IMD pathway responds to Gram-negative bacteria (32); however, both bacterial groups can induce the activation of Toll and IMD pathways in crustaceans (33). The expression of Cactus in the Toll pathway may be regulated in both cases of bacterial infection in crab, but whether or not the IMD pathway is regulated via Yki still remains unclear.

In summary, bacteria induced the high expression of DAZAP2 in crab, with DAZAP2 then binding to Sav, a core molecule in Hippo signaling, via the association of DAZAP2 and WW domains, to enhance the phosphorylation of Hpo that in turn can also bind with Sav. The tri-molecular complex of Hpo, Sav, and DAZAP2 suppressed the nuclear translocation of Yki, the expression of Cactus became inhibited, prompting the accelerated the nuclear translocation of dorsal from Toll signaling to up-regulate the AMPs'

expression, thus ultimately fostering innate immunity in crab.

Experimental Procedures

Animals and primary cultured hemocytes

Our experiments followed the protocol approved by the East China Normal University (ECNU) Animal Care and Use Committee (Protocol license number: AR2012/12017), in direct accordance with the Ministry of Science and Technology (People's Republic of China) animal care guidelines. Healthy *E. sinensis* crabs (100 ± 10 g each for adults and 15 ± 2 g each for naïve crabs, non-antibiotic or antifungal feed) were obtained from the Songjiang aquatic farm (Shanghai, China). After quickly transferring them to the Biological Experiment Station at ECNU, all crabs were maintained in filtered and aerated freshwater with plenty of oxygen provided, and fed daily a commercial formulated diet containing no antibiotics.

Primary cultures of *E. sinensis* hemocyte were generated following established techniques (34). From adult crab, isolated hemocytes were gently re-suspended in Leibovitz's L-15 medium (Sigma-Aldrich, Santa Clara, USA) supplemented with 1% antibiotics (10 000 U/ml penicillin, 10 000 μg/ml streptomycin [Gibco, Waltham, USA]) and 0.2 mM NaCl (676 ± 5.22 mOsm/kg), at a pH 7.20–7.40; hemocytes were then counted with an automated cell counter (Invitrogen Countess, Waltham, USA) before seeding 4 ml (1×10^6 cells/ml) each into 60-mm dishes.

Immune stimulation and sample collection

Staphylococcus aureus and *Vibrio parahemolyticus* were obtained from the National Pathogen Collection Center for Aquatic Animals (respectively, stock no. BYK0113 and BYK00036; Shanghai Ocean University, Shanghai, China). Each bacterial species was cultured, collected, and re-suspended in sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 [pH 7.4]).

Their counts were determined by plating the diluted suspension onto agar plates.

For the *in vitro* bacterial stimulation, cultured *S. aureus* or *V. parahemolyticus* (1×10^7 microbes per dish, 50 μl) was added separately to the hemocyte-cultured dish; sterile PBS (50 μl) was the control. Total RNA was collected from hemocytes at specific times since stimulation. Three or more crabs were used per sample. First-strand cDNA was synthesized using a Reverse Transcriptase Kit (Takara, Osaka, Japan), by following the manufacturer's instructions.

For the *in vivo* bacterial infection, *S. aureus* or *V. parahemolyticus* (1×10^8 CFU per crab, 200 μl) was injected into the hemolymph from the non-sclerotized membrane of the crab's posterior walking leg; the control consisted of sterile PBS (200 μl). Total RNA was collected from hemocytes at specific times since infection. Three or more crabs were used per sample.

Genomic sequencing, assembly, annotation, and phylogenetic analysis

Total DNA was extracted from the muscle of healthy Chinese mitten crabs (*E. sinensis*), then sent to the Novogene Company (Beijing, China) for whole genome sequencing on Illumina HiSeq 2000 and PacBio platforms (*unpublished*). Among this genomic data, we identified a fragment that containing a full open-reading frame encoding a protein with DAZAP2; this was designated as *Es*DAZAP2. Its full-length cDNA was amplified by a pair of gene-specific primers, and then re-sequenced to confirm the accuracy of the sequences. A similarity analysis was carried out using Clustalx from EMBL, with signal peptides and domain architecture predicted respectively by the SignalP v4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) and SMART (http://smart.embl-heidelberg.de/). An unrooted phylogenic tree was constructed based on the deduced amino acid sequences of *Es*DAZAP2 and other known DAZAP2 sequences by the neighbor-joining (NJ) algorithm using MEGA v6.0 software. To derive a

confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

RNA interference assay

All cDNA fragments of *Es*DAZAP2, *Es*Dorsal, *Es*Hpo, and *Es*Yki were PCR-amplified, by using primers linked to the T7 promoter (Table 1), to then serve as templates for producing siRNA with an *in vitro* T7 Transcription Kit (Fermentas, Burlington, Canada). For the control, we used GFP siRNA purchased from the GenePharma Company (Shanghai, China). For the *in vitro* RNAi, the siRNA was dissolved in RNase-free water and transfected into *E. sinensis* primary-cultured hemocytes by using Lipofectamine 3000 (Thermo Fisher, Waltham, USA) with 10-nM final concentration. For the *in vivo* RNAi, the siRNA (1 μg/g) was injected into crab hemolymph (of the non-sclerotized membrane of the posterior walking leg). To extend the RNAi effect, a 24 h after the first injection a second injection was administered to each crab. To determine RNAi efficiency, real-time RT-PCR was used with dsGFP RNA as the control. After setting up the RNAi assay, *S. aureus* or *V. parahemolyticus* were used to stimulate gene-silenced hemocytes *in vitro*, or injected into gene-silenced crabs *in vivo*.

Gene expression profile analysis

Expression levels of *Es*DAZAP2, *Es*Dorsal, *Es*Hpo, *Es*Yki, *Es*Cactus and various AMPs in the differently-treated hemocytes were determined with quantitative RT-PCR (qRT-PCR; primers showed in Table 1), by using CFX96^{TM} Real-Time System (Bio-Rad, Hercules, USA) and SYBR Premix Ex Taq (Tli RNaseH Plus; TaKaRa, Osaka, Japan). The following reaction conditions were used: 94°C for 3 min, then 40 cycles at 94°C for 10 s and 60°C for 1 min, followed by melting from 65°C to 95°C. The gene expression levels were derived by the $2^{-\Delta\Delta CT}$ calculation and normalized (to the control group). Three independent experiments were performed, with results given by their mean ± SD.

Assessment of crab survival and bacterial clearance assay

Crabs were randomly divided into two groups, each with 30 animals. Each crab in the first or second group was injected with *Es*DAZAP2 siRNA or 15 μg of GFP siRNA, respectively. After *Es*DAZAP2 was knocked down by the siRNA injection, all crabs were injected with *S. aureus* or *V. parahemolyticus* (1×10^9 CFU per crab, 200 μl). Dead crabs were counted daily in each group, and survival tabulated over 5 days. Three days after the bacterial injections, each crab's hemolymph was collected, diluted, and cultured overnight on solid LB plates. Bacterial colonies per plate were then counted.

Western blot

Protein samples were obtained from hemocytes by using the Coomassie Plus protein assay Reagent (Thermo Fisher, Waltham, USA). Whole-cell lysates were obtained by lysing cells with an RIPA buffer containing a mixture of protease and phosphatase inhibitors (Roche, USA), with protein concentration quantified by a Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, USA). Next, the protein was separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane and blocked for 1–2 h with 3% nonfat milk in a Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 8.0, 150 mM NaCl). This was incubated with 1:200 diluted antiserum against the proteins of interest, namely *Es*p-Hpo, *Es*Hpo, V5, His, and Actin—after confirming these antibodies generate non-specific signals—in TBS with 3% nonfat milk for 3 h. After washing the membrane three times in TBS, an alkaline phosphatase-conjugated goat anti-rabbit/mouse IgG (1:10 000 diluted in TBS) was added to it, then incubated with for 3 h, after which unbound IgG was washed away. The membrane was dipped into the reaction system and visualized in dark by 4-chloro-1-naphthol oxidation for 5 min. Antibodies recognizing the phosphorylated forms and total protein of Hpo were purchased from Cell Signaling Technology (Danvers, USA). Antibodies recognizing the total protein of V5, His, and Actin were purchased from Abcam (Cambridge, UK). All

images were collected using an Odyssey CLx (LI-COR, Lincoln, USA).

Immunocytochemical staining

To investigate the cellular translocation of Dorsal, Relish, Stat92E, and Yki in crab hemocytes, immunocytochemical staining was used as described in a previous study (35), with a few minor modifications. Briefly, pretreated hemocytes were blocked with 3%-bovine serum albumin (BSA) for 30 min at 37°C, then hemocytes were incubated overnight at 4 °C with the corresponding antibody (1:100 in blocking buffer). After washing with PBS, the hemocytes were incubated with 3% BSA for 10 min, followed by the addition of the second antibody: goat anti-mouse Alexa Fluor 488 (1:1000 dilution in 3% BSA). This reaction was maintained in the dark for 1 h at 37°C, then washed with PBS. Hemocytes were stained with 4' 6-diamidino-2-phenylindole dihydrochloride (DAPI, AnaSpec Inc., San Jose, USA), for 10 min at room temperature, and then washed again. All stained hemocytes were then observed under a Revolve Hybrid Microscope (Echo, USA).

Yeast two-hybrid library screening

The cDNA library for yeast two-hybrid (Y2H) experiments was built by OE BioTech (Shanghai, China), by cloning the cDNA synthesized from the mRNAs of the crab hemocytes into a prey vector, pGADT7 (Takara, Osaka, Japan). The full-length coding sequence of DAZAP2 was PCR-amplified by primers (Table 1) and inserted into the bait vector pGKBT7 (Takara, Osaka, Japan). Then the recombinant bait plasmid was transformed into the yeast Y2H Gold strain and tested for auto-activation and toxicity. Interactions between prey and bait proteins were confirmed using the Matchmaker™ Gold Yeast Two-Hybrid System (Clontech, Osaka, Japan), by following the manufacturer's instructions. Positive clones were sequenced to confirm the prey could encode the reading frame containing the GAL4 DNA activation domain. Positive (pGBKT7-53/pGADT7-T) and negative

(pGBKT7-Lam/pGADT7-T) controls were also prepared.

Co-immunoprecipitation

To confirm the interaction between DAZAP2, Hpo, and SAV *in vivo*, S2 cells were cultured for co-immunoprecipitation assays following previously reported methods. Briefly, *Drosophila* S2 cells were first seeded in 24-well plates and then cultured overnight at 28°C in Schneider's insect medium (Sigma, Santa Clara, USA) supplemented with 10% FBS (Life Technologies, Waltham, USA). After 24 h, cells were co-transfected with pAc5.1-DAZAP2-His, pAc5.1-Hpo-His, pAc5.1-Sav-V5, and pAc5.1-Sav without the WW domain-V5, pAc5.1-Sav without SARAH domain-V5, respectively, by using the Lipofectamine 3000 transfection reagent (Thermo Fisher, Waltham, USA) according to the manufacturer's instructions. At 36 h post-transfection, the S2 cells were washed twice with PBS before their proteins were extracted with an RIPA buffer, followed by centrifugation at 14 000 × *g* for 10 min. The supernatant, harboring a pool of different tag (His or V5)-containing proteins, was pre-cleared with 30 µl of Protein A beads for 40 min at 4°C with shaking. Next, the mixture was centrifuged at 12 000 × *g* for 10 min to remove the beads, with the ensuing supernatant incubated with 10 µg of His and V5 antibodies overnight at 4°C, under a gentle rotation. Protein A beads were then added to the mixture, which was incubated for 1 h at 4°C to capture the antibodies. The beads were collected via centrifugation and washed with PBS, then re-suspended in the SDS-PAGE loading buffer for separation by SDS-PAGE and the Western blot analysis (using the His or V5 antibody). To confirm the tri-molecular complex of Hpo, Sav and DAZAP2 in crab, 500 µl of protein from hemocytes in *E. sinensis* was incubated with *Es*Hpo, *Es*Sav, or *Es*DAZAP2 antibody overnight at 4 °C. Protein A/G magnetic beads for IP were added and incubated 3 h to capture the antigen–antibody complexes at 4 °C. The beads were collected via

centrifugation and washed with PBS three times. The mixture was resuspended with SDS-PAGE sample loading buffer and boiled at 100 °C for 5 min. The sample was centrifuged again and analyzed by Western blotting. Purified rabbit IgG was used as a control. All images were collected using an Odyssey CLx (LI-COR, Lincoln, USA).

Statements:

The authors declare that they have no conflicts of interest with the contents of this article. All authors agree with the submission. This work has not been published or submitted for publication elsewhere, either completely or in part, or in another form or language. No material has been reproduced from another source. All experiments using invertebrate animals were approved by the local authorities.

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Figure legends

Figure 1. Sequence information and expression pattern of DAZAP2. (A) The cDNA and amino acid sequences of DAZAP2. The sequence was subjected to an online SMART analysis. The SH2 (Yxxψ) and SH3 (PxψP) domain-binding motifs are respectively double and single underlined, the domain is boxed, and the DAZAP2 domain is darkened. **(B)** The DAZAP2 domain and the length of its amino acid sequences. **(C)** Phylogenetic analysis of DAZAP2 and some representative proteins from vertebrate and invertebrate species. The neighbor-joining phylogenetic tree was built in MEGA v6 (n = 1000 bootstraps). **(D)** Tissue distribution of DAZAP2. RNA samples were extracted from healthy *Eriocheir. sinensis* crabs, and DAZAP2 expression studied by RT-PCR (b-actin was the internal reference). Each sample was from at least three crabs, and the data are representative of three independent repeats. **(E-F)** Expression profiles of DAZAP2 mRNA in crab hemocytes after their infection with *Staphylococcus aureus* (E) *and Vibrio parahemolyticus* (F). RNA was extracted at each time point. qRT-PCR was used to check the expression of DAZAP2 in each sample, with b-actin as the reference. Shown as the mean ± SD. Three independent repeats were performed (≥ 5 crabs per sample). ** $p < 0.01$ (Student t test).

Figure 2. Protecting the crab host from bacterial infection by DAZAP2. (A) Effects of RNAi on DAZAP2. DAZAP2 siRNA (siDAZAP2) was designed to knock down DAZAP2 expression, and its expression in crab hemocytes determined 24 h post-injection with siDAZAP2, by qRT-PCR (siGFP = control). Shown are mean ± SD from three independent repeats (≥ 3 crabs per sample). **(B-C)** Knockdown of DAZAP2 led to crab death. Crabs were each injected with 15 ug of siDAZAP2 (siGFP = control) and their 5-day survival post-*S. aureus* (B) and -*V. parahemolyticus* (C) infection recorded from three independent repeats ≥ 10 crabs per sample. **(D-E)** RNAi of DAZAP2 increased bacterial proliferation in crabs treated as described above. From each, the hemolymph was drawn at day 3 post-*S. aureus* (D) and -*V. parahemolyticus* (E) infection and plated onto agar plates for bacterial counting. **(F-G)**

Bacteria induced high expression of antimicrobial peptides (AMPs). Hemocytes were stimulated by *S. aureus* (F) and *V. parahemolyticus* (G), with PBS as the control. **(H-I)** DAZAP2 regulates AMPs expression. *S. aureus* (H) and *V. parahemolyticus* (I) were used to stimulate DAZAP2-silenced hemocytes *in vitro* (siGFP = control). For panels **(F)** though **(I)** qRT-PCR was used to determine AMPs' expression at 12 h post-stimulation, and shown are the mean ± SD from three independent repeats (≥ 5 crabs per sample). ** $p < 0.01$ (Student t test).

Figure 3. DAZAP2 affects the Toll signaling pathway. (A) DAZAP2 influenced Dorsal translocation from cytoplasm into the nucleus. Immunocytochemical staining detected Dorsal (left), Relish (middle), and Stat92E (right) translocations in DAZAP2-silenced hemocytes post-*S. aureus* and -*V. parahemolyticus* stimulation. The green fluorescence signal indicated the target protein's distribution, while blue indicated the nuclei of hemocytes stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) (siGFP with bacterial stimulation = control). The data shown is from three independent repeats. **(B)** Effects of RNAi on Dorsal. Dorsal siRNA (siDorsal) was designed to knock down Dorsal expression in crab hemocytes, with expression determined 24 h after transfection with siDorsal, by qRT-PCR (siGFP = control). Shown are the mean ± SD from three independent repeats (≥ 3 crabs per sample). **(C-D)** Dorsal regulates AMPs' expression. *S. aureus* (C) and *V. parahemolyticus* (D) were used to stimulate Dorsal silenced hemocyte *in vitro* (siGFP = control); qRT-PCR was used to determine AMPs' expression at 12 h post-stimulation. Shown are the mean ± SD from three independent repeats (≥ 5 crabs per sample). Different lowercase letters indicate significantly different means, at $p < 0.01$ (one-way ANOVA) or ** $p < 0.01$, (Student t test).

Figure 4. DAZAP2 interacts with Sav. (A) Two-hybrid principle (Y2H): Two proteins are expressed separately, with a bait protein fused to the Gal4 DNA-binding domain (BD) and the prey protein fused to the Gal4 transcriptional activation domain

(AD). **(B-C)** Tests for auto-activation and toxicity of pGBKT7-DAZAP2 (B) and pGADT7-Sav (C). **(D-E)** Confirmation of positive interactions between DAZAP2 and Sav, based on the Y2H (D) and the *in vivo* co-immunoprecipitation assays (E). Positive (pGBKT7-53/pGADT7-T) and negative (pGBKT7-Lam/pGADT7-T) controls were used in Y2H. SD: medium comprised of a nitrogen base, a carbon source, and a DO supplement for yeast. DO: dropout (supplement or solution), a mixture of specific amino acids and nucleosides used to supplement the SD base to make the SD medium; the DO solutions are missing one or more of the nutrients required by untransformed yeast to grow on the SD medium. DDO: double-dropout medium, SD/–Leu/–Trp. DDO/X/A: double-dropout medium, SD/–Leu/–Trp supplemented with X-a-Gal and Aureobasidin A. QDO: quadruple-dropout medium, SD/–Ade/–His/–Leu/–Trp. QDO/X/A: quadruple-dropout medium, SD/–Ade/–His/–Leu/–Trp supplemented with X-a-Gal and Aureobasidin A.

Figure 5. DAZAP2 regulates the Hippo signaling pathway. (A) The upper panes show bacteria-induced Hpo phosphorylation. Western blot detected enhanced Hpo phosphorylation post-*S. aureus* and *V. parahemolyticus* stimulation at 0.5 h and 1 h in crab hemocytes, with b-actin as reference; bottom panels represent the statistical analysis of phosphorylated Hpo in the upper panels. **(B)** Bacteria inhibit Yki translocation from cytoplasm into nucleus in hemocytes, detected by immunocytochemical staining at 0.5 h and 1 h post-*S. aureus* and -*V. parahemolyticus* stimulation. The green fluorescence signal indicated the distribution of Yki; blue indicated the nuclei of hemocytes stained with DAPI (PBS = control). **(C)** Upper panes show that DAZAP2 regulates Hpo phosphorylation. A Western blot detected reduced Hpo phosphorylation post-*S. aureus* and -*V. parahemolyticus* stimulation at 0.5 h and 1 h in DAZAP2-silenced hemocytes (siGFP = control; β-actin was used as reference); the bottom panels represent a statistical analysis of phosphorylated Hpo in the upper panels. **(D)** DAZAP2 negatively regulates Yki translocation from cytoplasm into nucleus of DAZAP2-silenced hemocytes, detected by immunocytochemical

staining at 0.5 h post-*S. aureus* and -*V. parahemolyticus* stimulation (siGFP plus bacterial stimulation = control). Green fluorescence signal indicated the distribution of Yki; blue indicated the nuclei of hemocytes stained with DAPI. All data shown are from three independent repeats. Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$) analyzed by a Student's *t*-test.

Figure 6. Signal transduction of the Hippo pathway regulates AMPs' expression. **(A-B)** Effects of RNAi on Hpo and Yki. Hpo and Yki siRNA (siHpo and siYki) were designed to knock down Hpo and Yki expression in crabs, respectively. The expression of Hpo (A) and Yki (B) in hemocytes was determined 24 h after siRNA transfection, by qRT-PCR (siGFP = control). Shown are the mean ± SD from three independent repeats (≥ 3 crabs per sample). **(C)** Hpo negatively regulates Yki translocation from the cytoplasm into nucleus. Immunocytochemical staining was used to detect Yki translocation at 0.5 h post-*S. aureus* and -*V. parahemolyticus* stimulation in Hpo-silenced hemocytes (siGFP plus bacterial stimulation = control). The green fluorescence signal indicated the distribution of Yki; blue indicated the nuclei of hemocytes stained with DAPI. The data are from three independent repeats. **(D-E)** Both Yki and DAZAP2 regulate Cactus expression. Yki- (D) and DAZAP2-(E) silenced hemocytes were treated with or without *S. aureus* and *V. parahemolyticus* stimulations, (siGFP = control). qRT-PCR was used to determine Cactus expression at 12 h post-stimulation. Shown are the mean ± SD from three independent repeats (≥ 5 crabs per sample). **(F-G)** Hpo regulate AMPs expression. *S. aureus* (F) and *V. parahemolyticus* (G) were used to stimulate Hpo silenced hemocyte *in vitro*, with siGFP as control. qRT-PCR was used to determine AMPs expression at 12 h post-stimulation. Shown are the mean ± SD from three independent repeats (≥ 5 crabs per sample). Different lowercase letters indicate significantly different means, at $p < 0.01$ (one-way ANOVA) or ** $p < 0.01$ (Student t test).

Figure 7. DAZAP2 and Hpo interact with different domain of Sav. (A) Illustration of the wild type and truncated Sav protein. **(B)** Interaction between DAZAP2 and Sav *in vivo*. **(C)** Interaction between Hpo and Sav *in vivo*. In both **(B)** and **(C)**, lysates from S2 cells were transiently transfected with pAc5.1-Hpo-His or together with pAc5.1-SAV-V5, or with pAc5.1-SAV lacking the WW domain-V5, or with pAc5.1-SAV lacking the SARAH domain-V5; all were subjected to immunoprecipitation with anti-His Ab or anti-V5 Ab, followed by a Western blot analysis that was applied to the input controls. (D) Tri-molecular complex of Hpo, Sav, and DAZAP2. A co-immunoprecipitation (IP) assay showed the interaction between Hpo, Sav, and DAZAP2 in crab hemocytes. IgG was used as a control. Data are from two independent repeats.

Figure 8. Schematic representation of DAZAP2-regulated antimicrobial activities in crab. Bacteria induced high levels of DAZAP2 expression in crab, with DAZAP2 subsequently binding to Sav, a core molecule in Hippo signaling, via the association of DAZAP2 and WW domains. This functioned to enhance the phosphorylation of Hpo, which in turn can also bind to Sav via the SARAH domain. Since the nuclear translocation of Yki was suppressed, the transcription of Cactus was inhibited, prompting the acceleration of dorsal nuclear translocation that induced by Toll signaling to up-regulate AMP expression.

Figure. 1

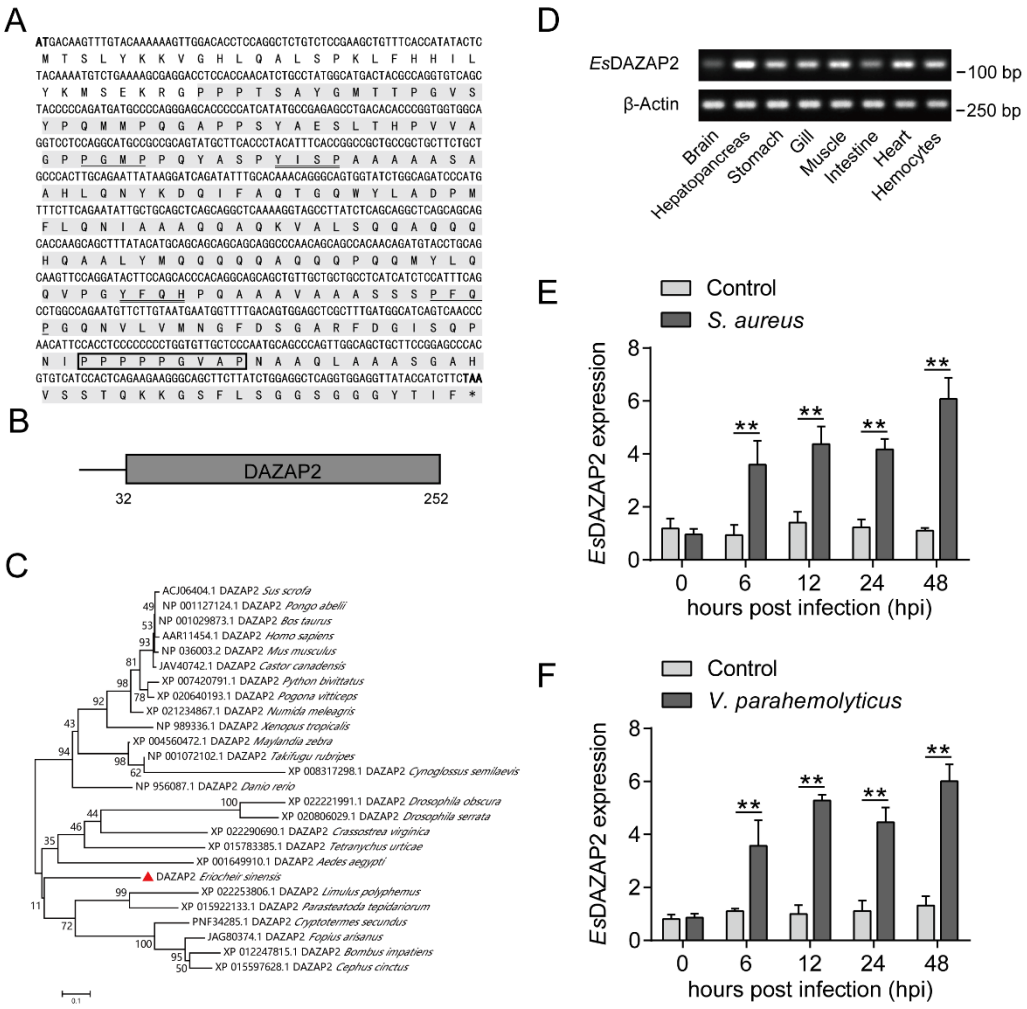


Figure. 2

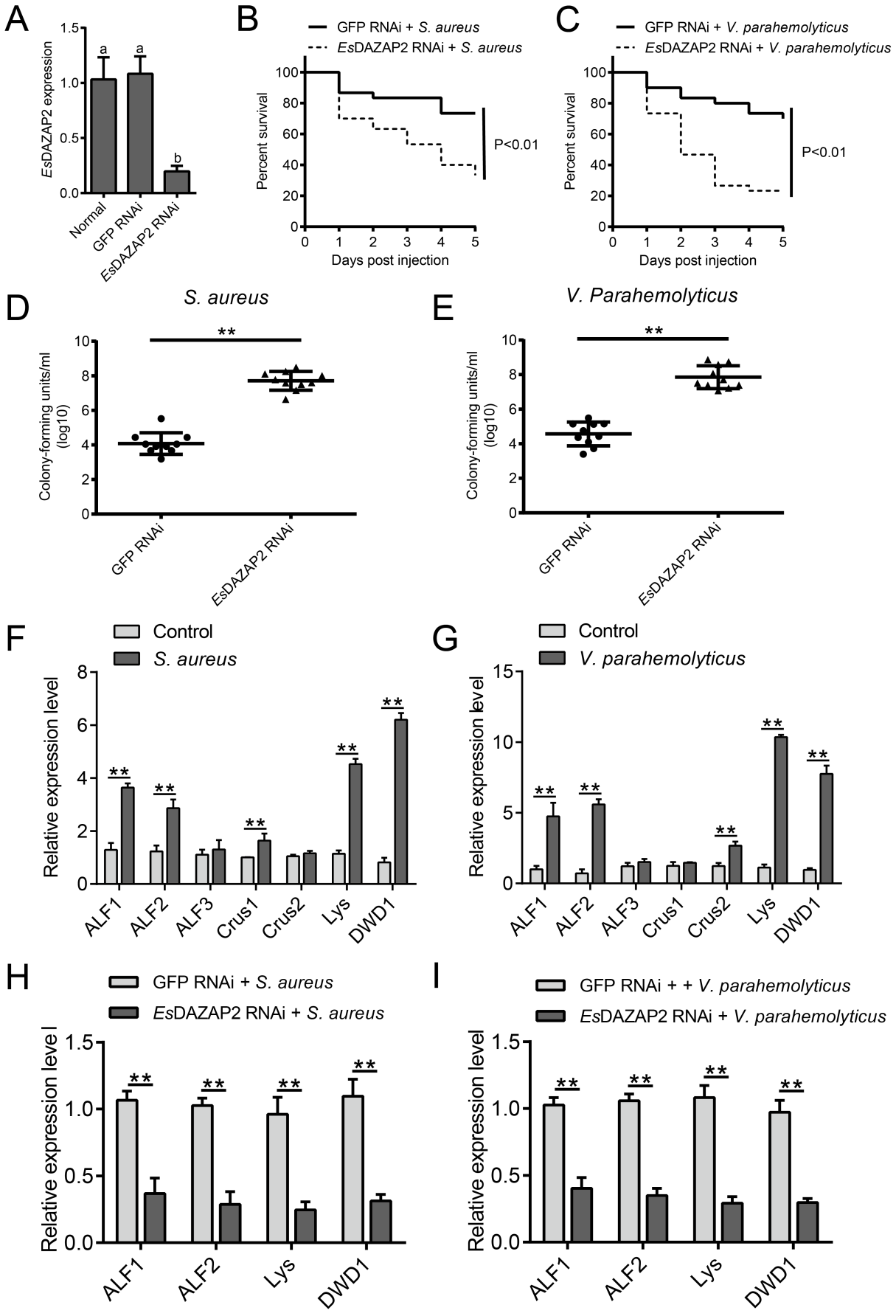


Figure. 3

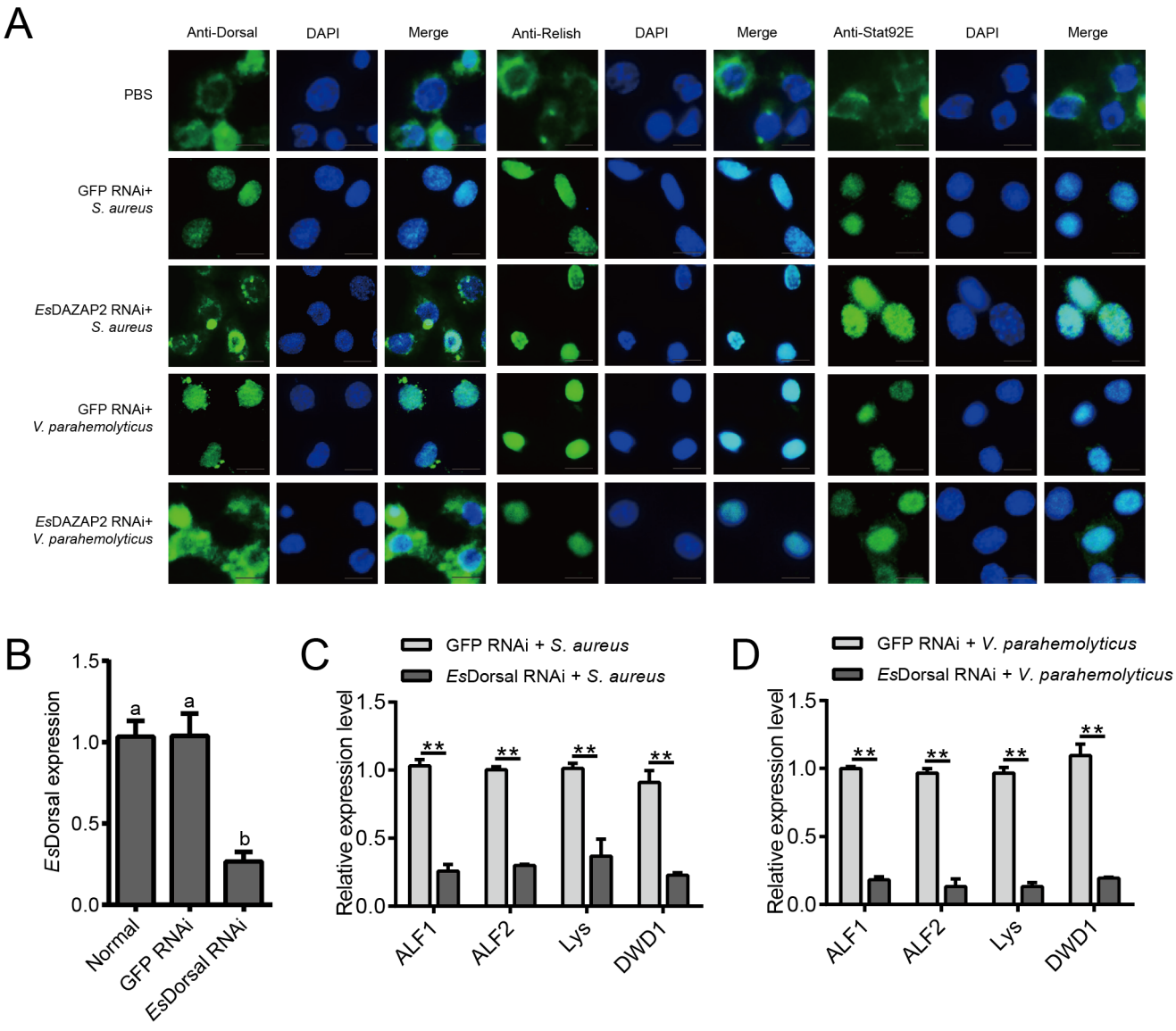
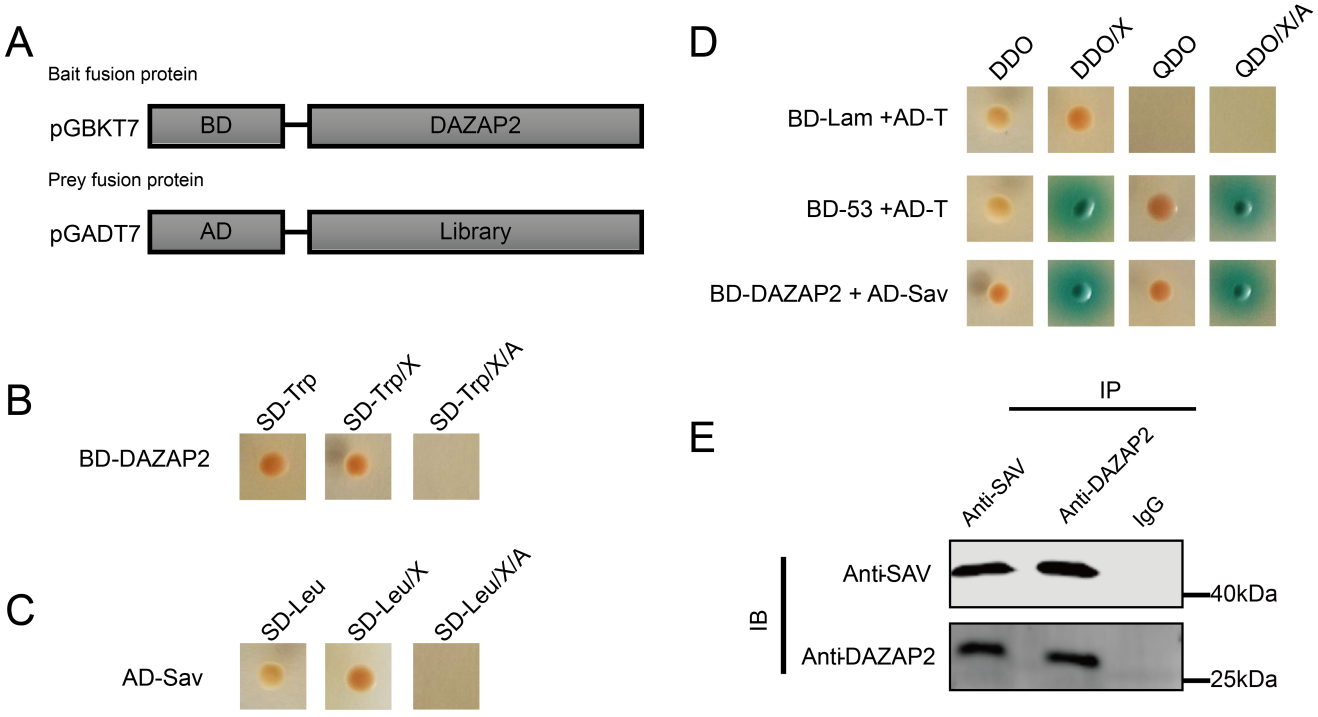


Figure. 4



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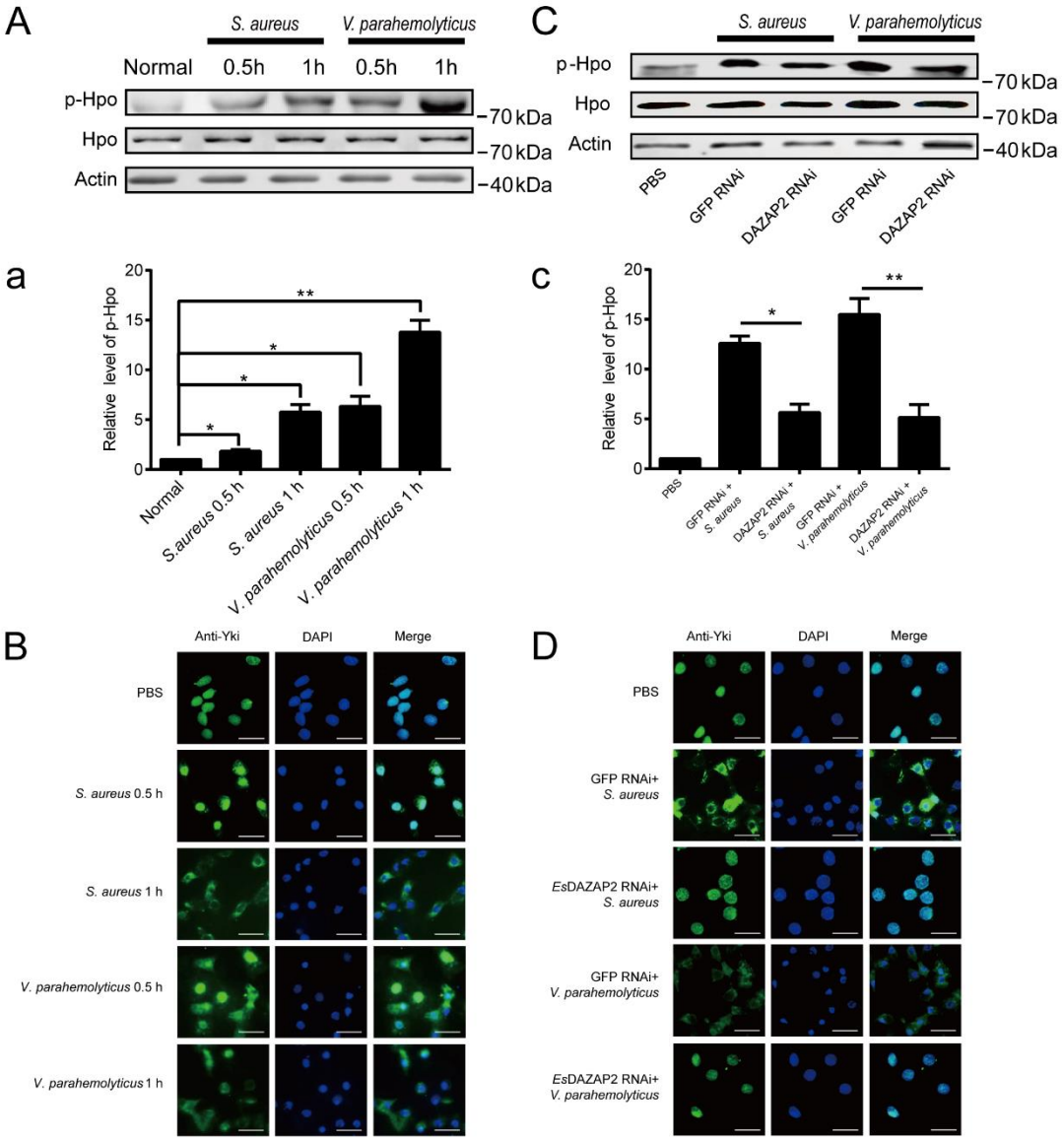

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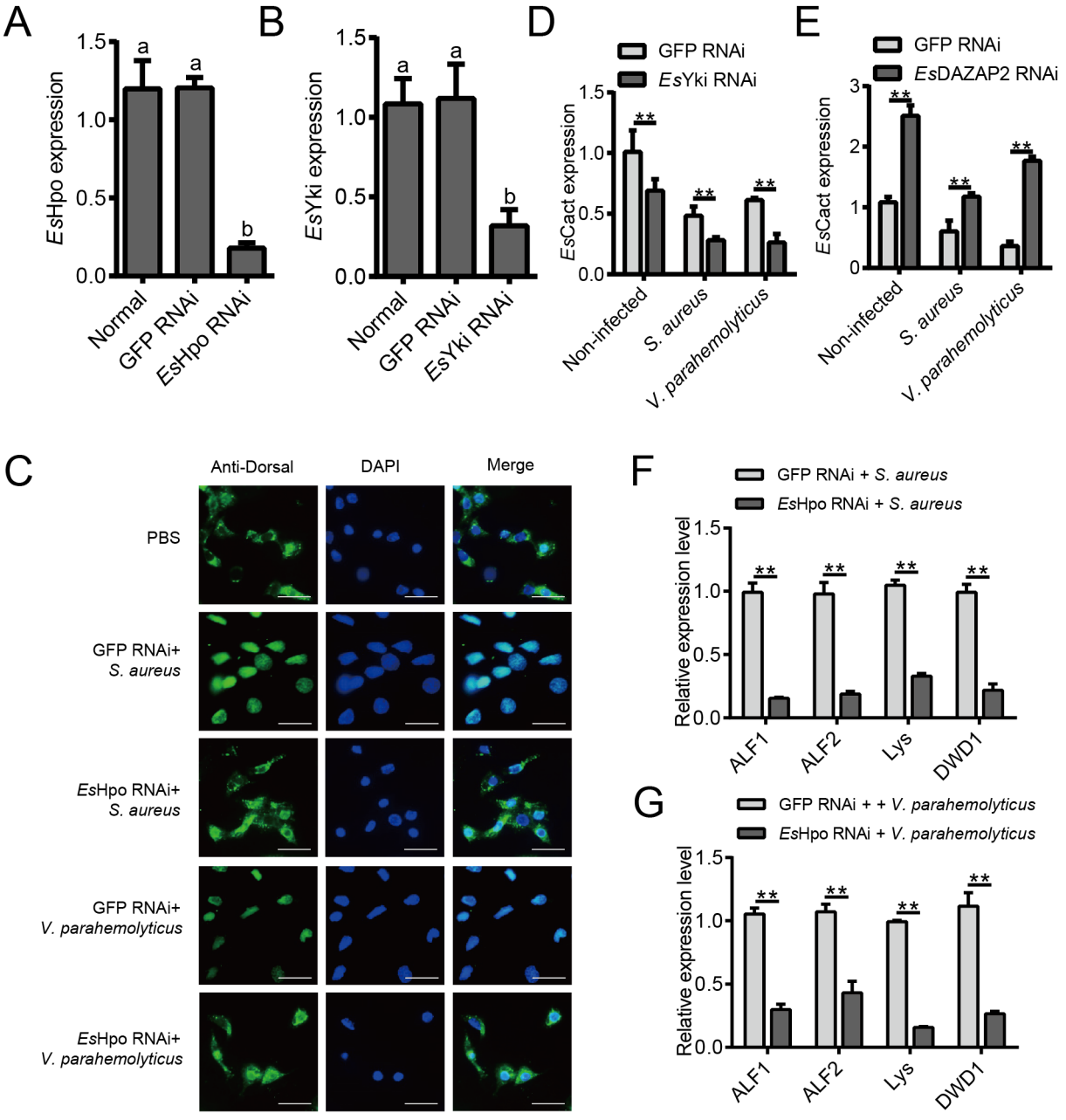


Figure. 7

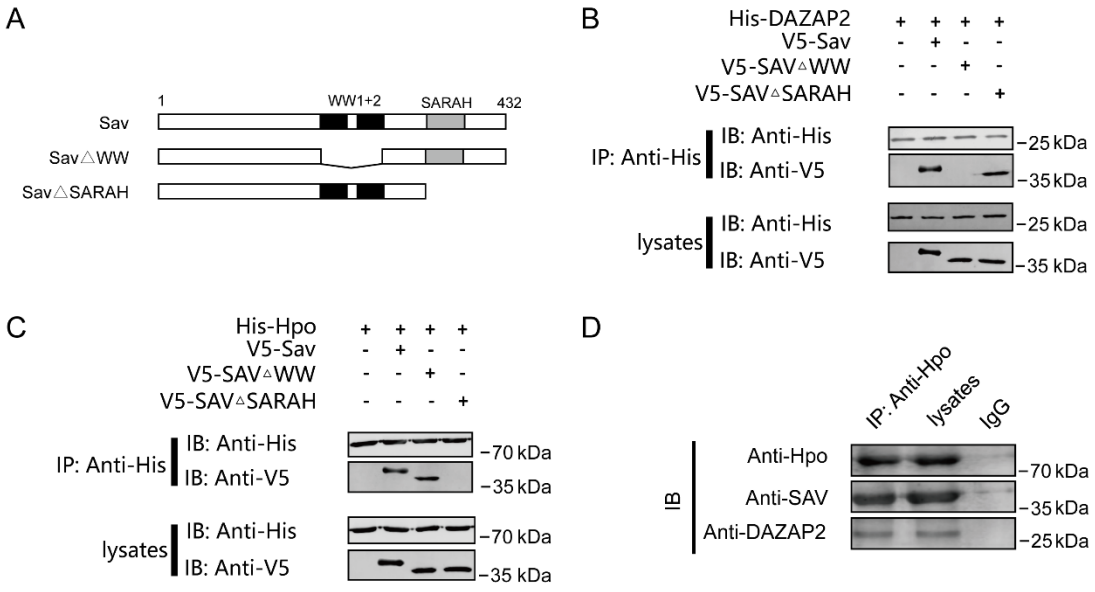
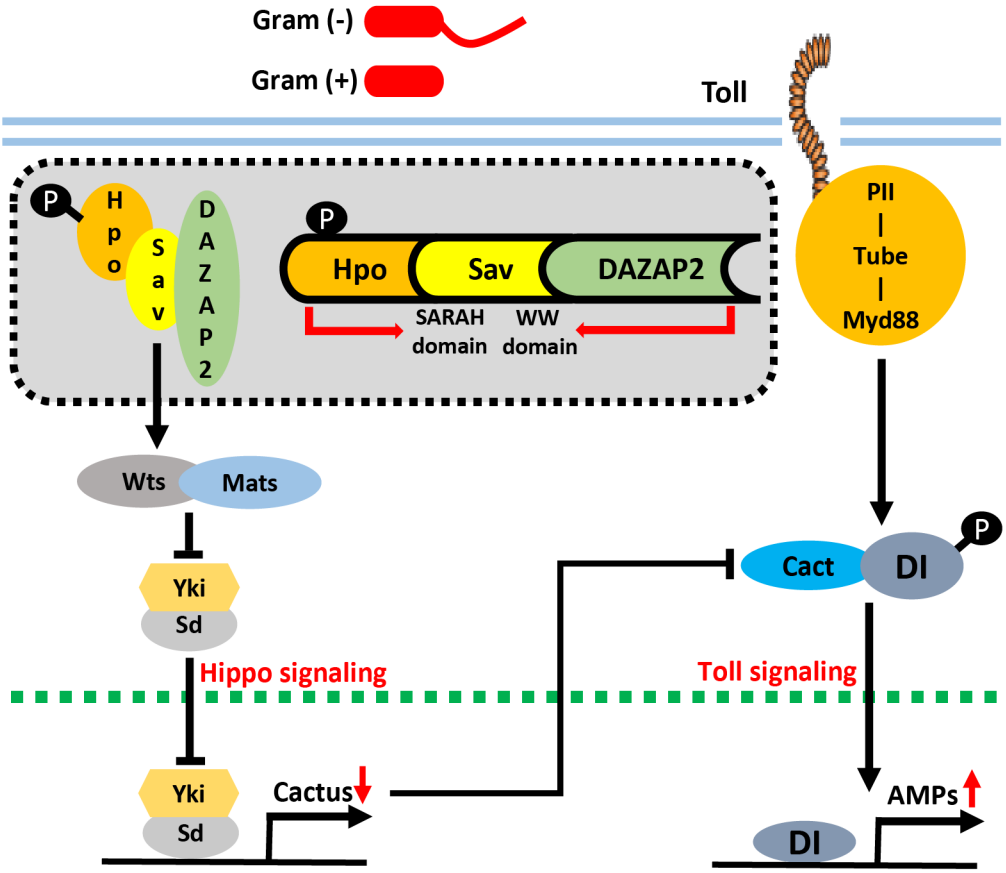


Figure. 8



Deleted in azoospermia-associated protein 2 regulates innate immunity by stimulating Hippo signaling in crab

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