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

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The Hippo-signaling pathway plays a critical role in both normal animal physiology and pathogenesis. Because 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 the 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 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 transcription 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.

Invertebrates, such as insects and crustaceans, rely solely on innate immunity for their immunological defense, and so they have evolved complex mechanisms to counter microbial infections [1]. Pioneering studies in *Drosophila* delineated two key signaling pathways, namely Toll and Imd,[4] 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 allows us to 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 suggests 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 nondevelopmental and nongrowth-related processes remain 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 IκB 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–producing mitochondria through Rac activation (16). Taken together, the above studies reveal critical roles of the Hippo pathway in antibacterial immunity through several different mechanisms (6, 15, 16). Nevertheless, how these mechanisms are coordinated in different environments and their regulators' involvement still await 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 (SH) 2- and SH3-binding motifs (17). DAZAP2 mRNA and protein expression are frequently down-regulated in multiple myeloma patients, whereas 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 down-regulated 4 (NEDD 4) (20). Moreover, a knockdown of DAZAP2 could both reduce the level of Wnt-signaling activity and 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 remain largely unknown in invertebrates.

The 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 reac-

tions 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 the 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 complementary 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 *E. 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-kDa 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. 1, *A* 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. 1*C*), consistent with classical zoological systematics. The *DAZAP2* gene was expressed in different tissues harboring hemocytes (Fig. 1*D*), which hinted at its role in crab physiology. To test whether DAZAP2 expression could be induced post-infection, *Staphylococcus aureus* (Fig. 1*E*) and *Vibrio parahemolyticus* (Fig. 1*F*) 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. 2*A*). This was followed by greatly reduced crab survivorship and significantly enhanced bacterial concentrations in the hemolymph since the infection with *S. aureus* (Fig. 2, *B* and *D*) or *V. parahemolyticus* (Fig. 2, *C* and *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. 2*F*) and *V. parahemolyticus* (Fig. 2*G*) 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 was knocked down in hemocytes *in vitro*; this significantly inhibited AMPs' expression after stimulation with either *S. aureus* (Fig. 2*H*) or *V. parahemolyticus* (Fig. 2*I*). Together, these results
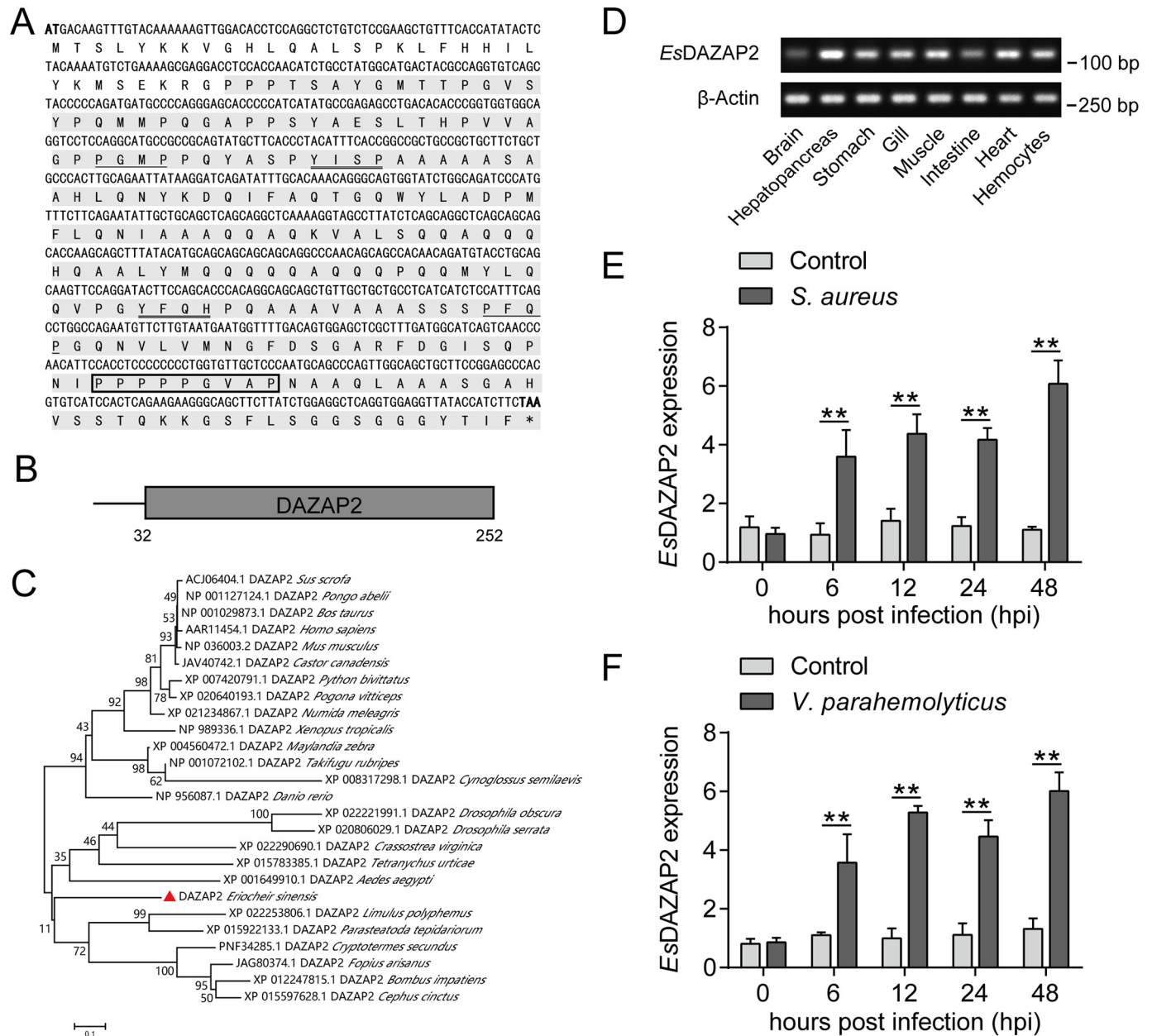
Figure 1. Sequence information and expression pattern of DAZAP2. *A,* cDNA and amino acid sequences of DAZAP2. The sequence was subjected to an on-line SMART analysis. The SH2 (YXXψ) and SH3 (PXψP) domain-binding motifs are *double* and *single underlined*, respectively; the domain is *boxed,* and the DAZAP2 domain is *shaded. B,* 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 version 6 (*n* = 1000 bootstraps). *D,* tissue distribution of DAZAP2. RNA samples were extracted from healthy *E. sinensis* crabs, and DAZAP2 expression was studied by RT-PCR (β-actin was the internal reference). Each sample was from at least three crabs, and the data are representative of three independent repeats. *E* and *F,* expression profiles of DAZAP2 mRNA in crab hemocytes after their infection with *S. aureus* (*E*) and *V. parahemolyticus* (*F*). RNA was extracted at each time point. qRT-PCR was used to check the expression of DAZAP2 in each sample, with β-actin as the reference. Shown are the means ± S.D. Three independent repeats were performed (≥5 crabs per sample). **, $p < 0.01$ (Student's *t* test).

demonstrated a critical involvement of DAZAP2 in antibacterial immune reactions.

DAZAP2-regulated AMP 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 the nucleus, which demonstrated it may regulate AMPs' expression via the Toll pathway. Going one step further, we effectively knocked down Dorsal
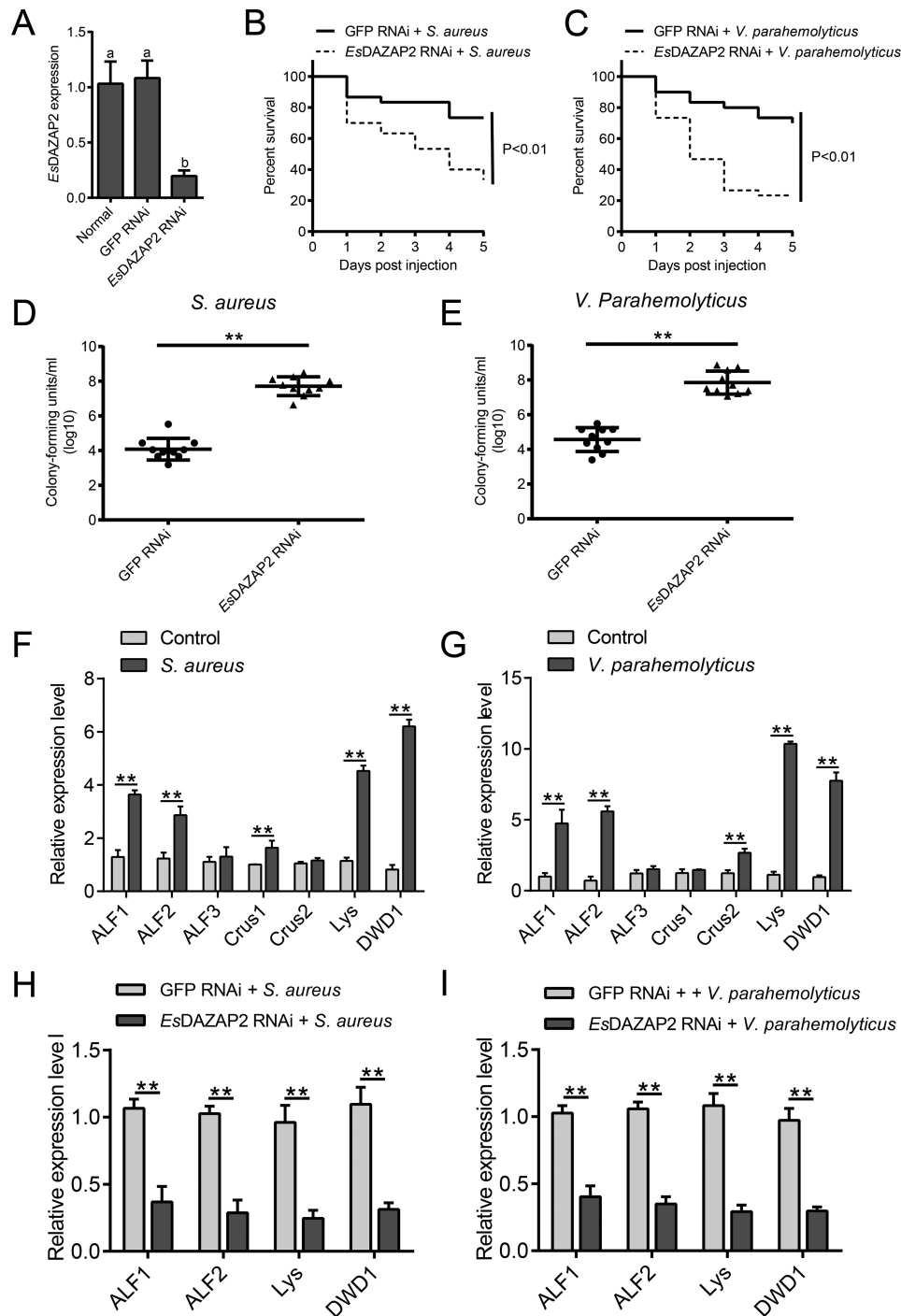
Figure 2. Protecting the crab host from bacterial infection by DAZAP2. *A,* effects of RNAi on DAZAP2. DAZAP2 siRNA (siDAZAP2) was designed to knock down DAZAP2 expression, and its expression in crab hemocytes was determined 24 h post-injection with siDAZAP2 by qRT-PCR (siGFP = control). Shown are means ± S.D. from three independent repeats (≥3 crabs per sample). *B* and *C,* knockdown of DAZAP2 led to crab death. Crabs were each injected with 15 μg of siDAZAP2 (siGFP = control), and their 5-day survival post-*S. aureus* (*B*) and post-*V. parahemolyticus* (*C*) infection was recorded from three independent repeats of ≥10 crabs per sample. *D* and *E,* RNAi of DAZAP2 increased bacterial proliferation in crabs treated as described above. From each crab, the hemolymph was drawn at day 3 post-*S. aureus* (*D*) and post-*V. parahemolyticus* (*E*) infection and plated onto agar plates for bacterial counting. *F* and *G,* bacteria induced high expression of AMPs. Hemocytes were stimulated by *S. aureus* (*F*) and *V. parahemolyticus* (*G*), with PBS as the control. *H* and *I,* DAZAP2 regulates AMPs expression. *S. aureus* (*H*) and *V. parahemolyticus* (*I*) were used to stimulate DAZAP2-silenced hemocytes *in vitro* (siGFP = control). *F–I,* qRT-PCR was used to determine AMPs' expression at 12 h post-stimulation, and shown are the means ± S.D. from three independent repeats (≥5 crabs per sample). **, $p < 0.01$ (Student's *t* test).

expression in hemocytes (Fig. 3*B*), which revealed the expression levels of AMPs regulated by DAZAP2 were significantly reduced in Dorsal-silenced hemocytes after infection with *S. aureus* (Fig. 3*C*) or *V. parahemolyticus* (Fig. 3*D*).

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, in order to
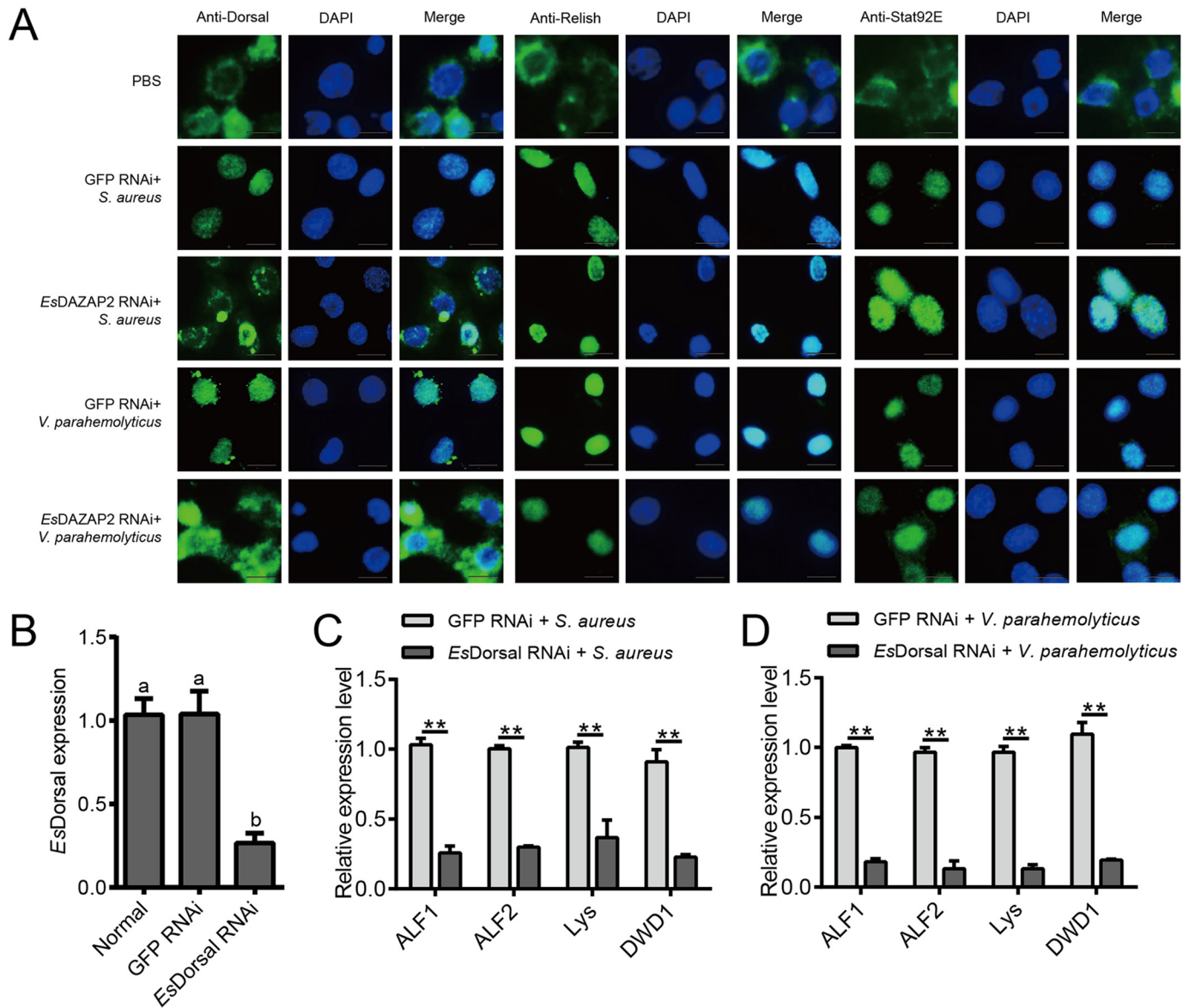
Figure 3. DAZAP2 affects the Toll-signaling pathway. *A*, DAZAP2 influenced Dorsal translocation from the cytoplasm into the nucleus. Immunocytochemical staining detected Dorsal (*left*), Relish (*middle*), and Stat92E (*right*) translocations in DAZAP2-silenced hemocytes post-*S. aureus* and post-*V. parahemolyticus* stimulation. The *green* fluorescence signal indicates the target protein's distribution, and the *blue* indicates the nuclei of hemocytes stained with DAPI (siGFP with bacterial stimulation = control). The data shown are 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 means ± S.D. from three independent repeats (≥3 crabs per sample). *C* and *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 means ± S.D. 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).

regulate Dorsal translocation and subsequent AMP expression. To identify such DAZAP2-interacting proteins and to elucidate the underlying molecular mechanism for AMPs' regulation, a 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. 4*A*), with the ensuing construct transformed into a yeast Y2H Gold strain. The pGBKT7–DAZAP2 bait protein did not autonomously activate the reporter genes and was confirmed as nontoxic (Fig. 4*B*). 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 gen-

uine 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. 4*C*). Further evidence confirming the physical interaction between DAZAP2 and Sav came from both the Y2H (Fig. 4*D*) and *in vivo* co-immunoprecipitation assays in crab hemocytes (Fig. 4*E*).

DAZAP2-regulated Hippo-signaling activation

Because of 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.
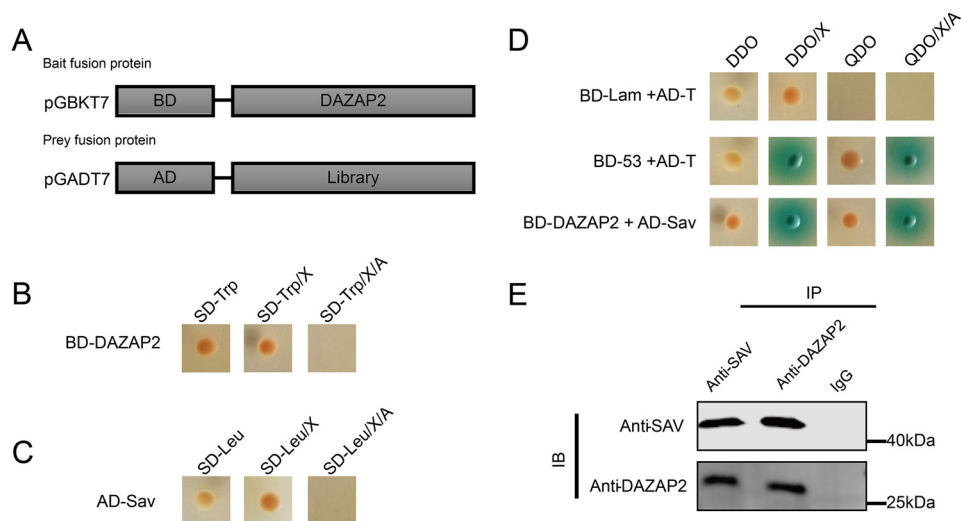
Figure 4. DAZAP2 interacts with Sav. *A,* Y2H principle (Y2H). Two proteins are expressed separately, with a bait protein fused to the Gal4 DNA-binding domain (*BD*), and the prey protein was fused to the Gal4 transcriptional activation domain (*AD*). *B* and *C,* tests for auto-activation and toxicity of pGBKT7–DAZAP2 (*B*) and pGADT7–Sav (*C*). *D* and *E,* confirmation of positive interactions between DAZAP2 and Sav, based on the Y2H (*D*) and the *in vivo* co-immuno-precipitation assays (*E*). Positive (pGBKT7–53/pGADT7-T) and negative (pGBKT7-Lam/pGADT7-T) controls were used in Y2H. SD: medium composed of a nitrogen base, a carbon source, and a dropout supplement for yeast. *DO* is dropout (supplement or solution), a mixture of specific amino acids and nucleosides used to supplement the SD base to make the SD medium; the dropout solutions are missing one or more of the nutrients required by untransformed yeast to grow on the SD medium. *DDO* is double-dropout medium; SD/−Leu/−Trp. DDO/X/A: double-dropout medium, SD/−Leu/−Trp supplemented with X-α-Gal and aureobasidin A. *QDO* is quadruple-dropout medium, SD/−Ade/−His/−Leu/−Trp. QDO/X/A is quadruple-dropout medium, SD/−Ade/−His/−Leu/−Trp supplemented with X-α-Gal and aureobasidin A. *IP* is immunoprecipitation; *IB* is immunoblot.

Hpo, Sav, and Yki were conserved among different species (Fig. S2). In *Drosophila*, it is known that Hpo becomes phosphory-lated after binding with Sav, which inhibits the Yki transloca-tion from the cytoplasm into the 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 and 1 h post-stimulation (Fig. 5*A*). Moreover, Yki did not reach the nucleus in hemocytes, after either *S. aureus* or *V. parahemolyti-cus* stimulation at 0.5 and 1 h (Fig. 5*B*). Given the key impor-tance 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. 5*C*). Moreover, most Yki were translocated from the cytoplasm into the nucleus in DASZAP2-silenced hemocytes following the *S. aureus* or *V. parahemolyticus* stimulation (Fig. 5*D*). Collec-tively, 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 activi-ties 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' expres-sion in crab, Hpo was knocked down in hemocytes (Fig. 6*A*), which clearly showed decreased Dorsal in the nucleus after the *S. aureus* and *V. parahemolyticus* stimulations (Fig. 6*C*). Because dorsal translocation is inhibited by Yki-regulated Cac-tus expression in *Drosophila*, we effectively knocked down Yki expression in hemocytes (Fig. 6*B*), and this significantly decreased the expression of Cactus after stimulations with

S. aureus and *V. parahemolyticus* (Fig. 6*D*). To study whether DAZAP2 regulates Cactus expression, DAZAP2 was knocked down in crab hemocytes, which led to a significant up-regulation of Cactus transcription following stimulation with *S. aureus* and *V. parahemolyticus* (Fig. 6*E*). Furthermore, those AMPs regulated by DAZAP2 had very significantly suppressed expression levels in Hpo-silenced hemocytes, after the *S. aureus* (Fig. 6*F*) and *V. parahemolyticus* (Fig. 6*G*) stimula-tions. These results demonstrated that Hippo signaling can also regulate AMPs' expression in crab.

DAZAP2 and Hpo interacted with different domains of Sav

The protein complexes 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 the same or a 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. 7*A*). 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. 7*B*). Conversely, Hpo interacted with the full-length Sav via the SARAH domain but not the WW domain (Fig. 7*C*). To study the relationship between Hpo, Sav, and DAZAP2, a co-IP assay was conducted in crab hemocytes, and the results demon-strated the interaction between these proteins *in vivo* (Fig. 7*D*). 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.
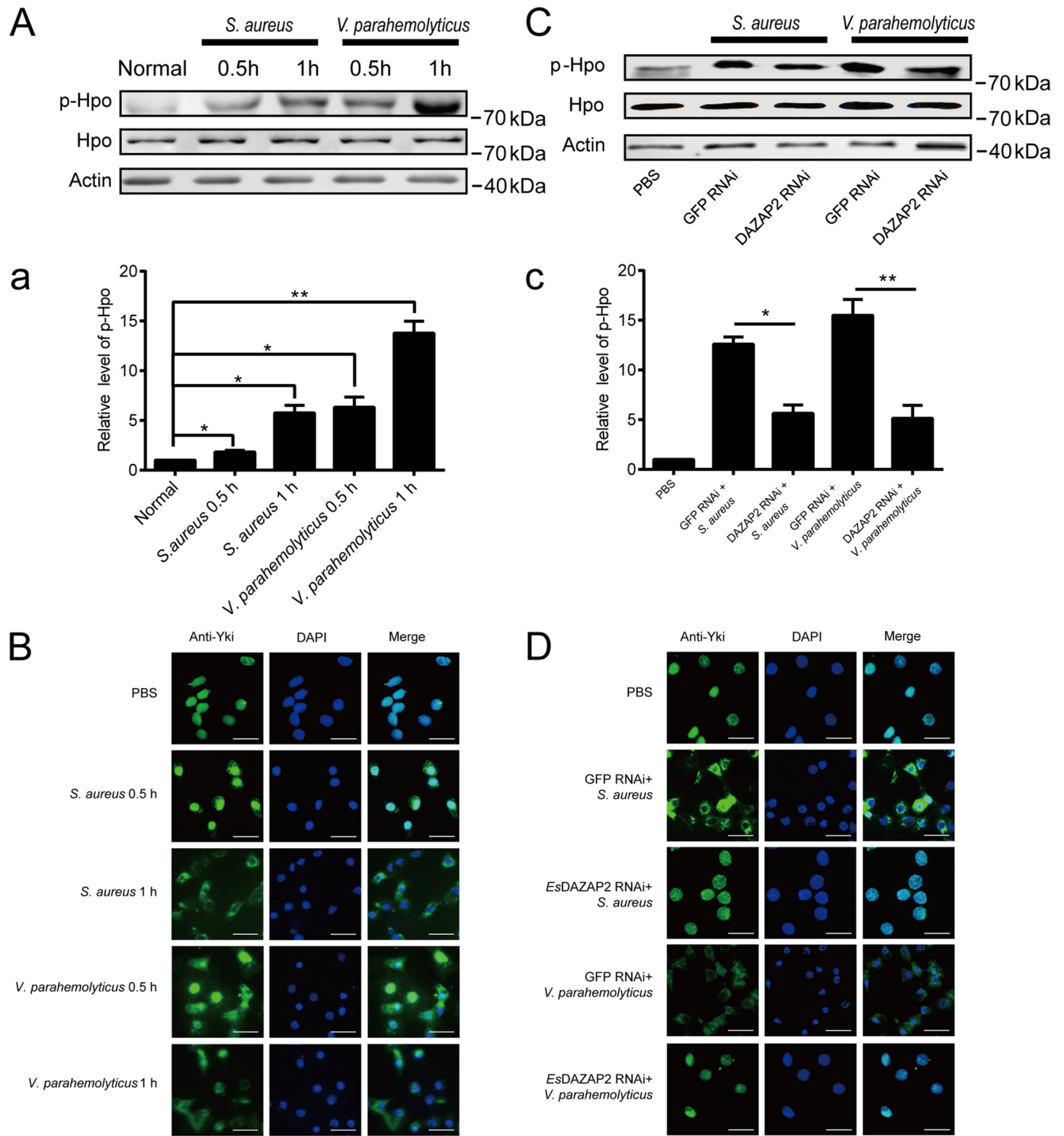
Figure 5. DAZAP2 regulates the Hippo-signaling pathway. *A, upper panels* show bacteria-induced Hpo phosphorylation. Western blotting detected enhanced Hpo phosphorylation post-*S. aureus* and post-*V. parahemolyticus* stimulation at 0.5 and 1 h in crab hemocytes, with β-actin as reference; *bottom panels* 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 and 1 h post-*S. aureus* and post-*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 panels* show that DAZAP2 regulates Hpo phosphorylation. Western blotting detected reduced Hpo phosphorylation post-*S. aureus* and post-*V. parahemolyticus* stimulation at 0.5 and 1 h in DAZAP2-silenced hemocytes (siGFP = control; β-actin was used as reference); *bottom panels* represent a statistical analysis of phosphorylated Hpo in the *upper panels*. *D*, DAZAP2 negatively regulates Yki translocation from the cytoplasm into the nucleus of DAZAP2-silenced hemocytes, detected by immunocytochemical staining at 0.5 h post-*S. aureus* and post-*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.
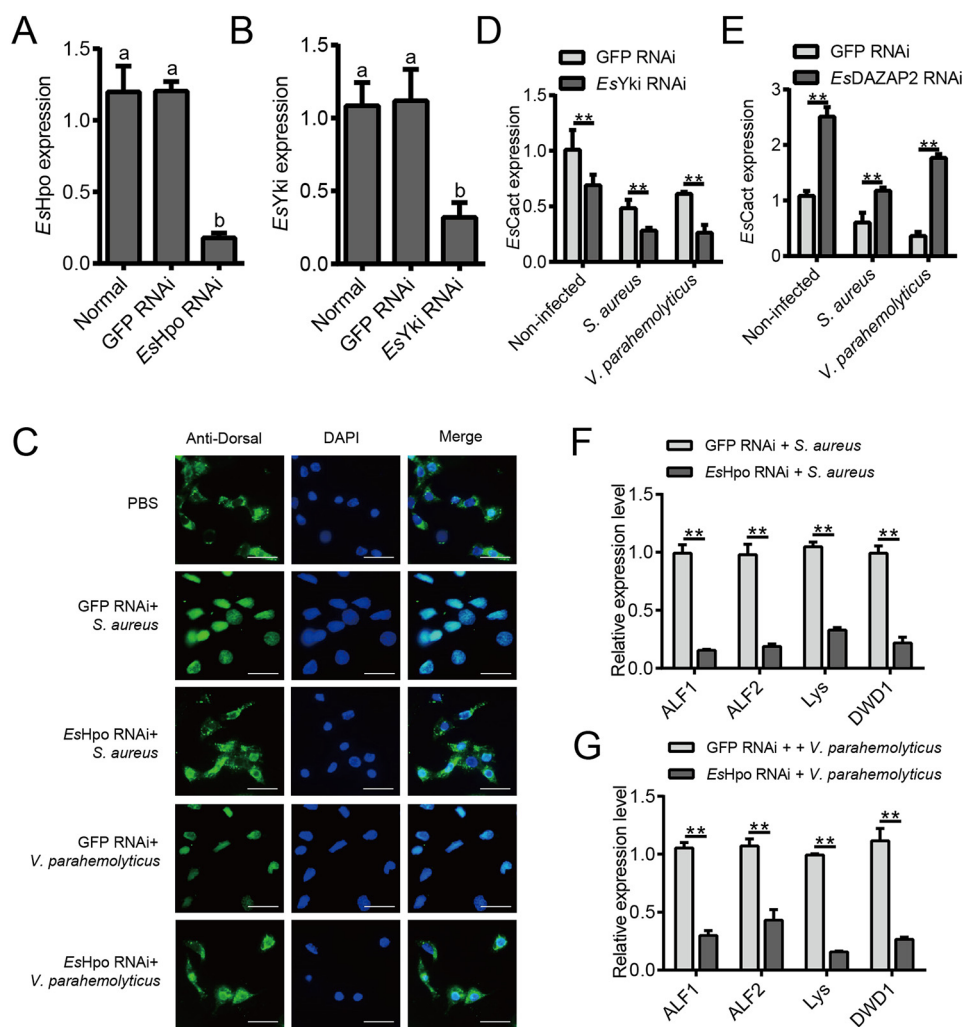
Figure 6. Signal transduction of the Hippo pathway regulates AMPs' expression. *A* and *B*, effects of RNAi on Hpo and Yki. Hpo and Yki siRNA (siHpo and siYki) were designed to knock down Hpo and Yki expression in crabs, respectively. The expression of Hpo (*A*) and Yki (*B*) in hemocytes was determined 24 h after siRNA transfection, by qRT-PCR (siGFP = control). Shown are the means ± S.D. 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 post-*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* and *E*, both Yki and DAZAP2 regulate Cactus expression. Yki-silenced (*D*) and DAZAP2-silenced (*E*) 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 means ± S.D. from three independent repeats (≥5 crabs per sample). *F* and *G*, Hpo regulates AMP 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 means ± S.D. 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).

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). The research in *Drosophila* that 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. sinen-sis* 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 (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
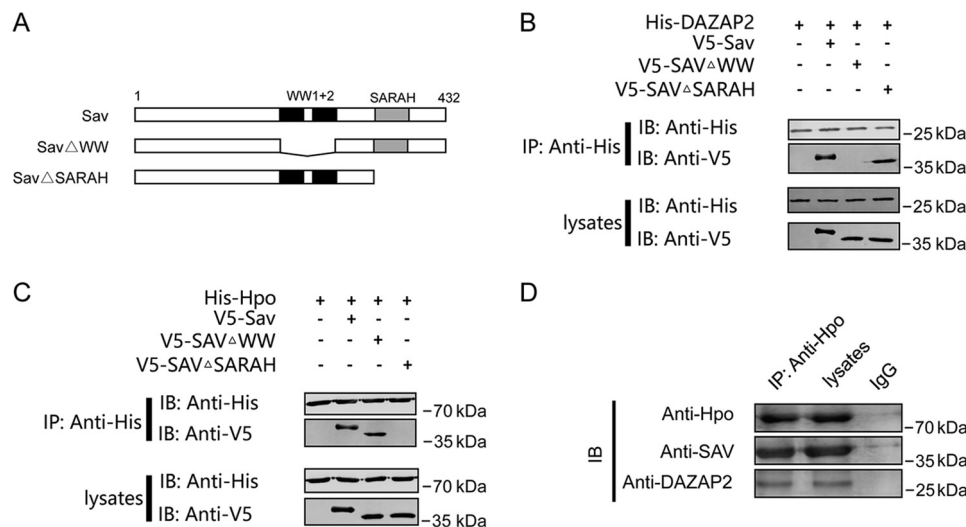
Figure 7. DAZAP2 and Hpo interact with different domain of Sav. *A,* illustration of the WT and truncated Sav protein. *B,* interaction between DAZAP2 and Sav *in vivo. C,* interaction between Hpo and Sav *in vivo.* In both *B* and *C,* lysates from S2 cells were transiently transfected with pAc5.1-Hpo-His 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. *IB* is immunoblot.

the AMPs' expression, bacterial clearance, and host crab survivorship, which spurred us to explore the immune mechanisms of *Es*DAZAP2. Because of 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 molecule in the 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 cross-talk 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 the 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, and the expression of Cactus became inhibited, prompting the accelerated nuclear translocation of Dorsal from Toll signaling to up-regulate the AMPs' expression, thus ultimately fostering innate immunity in crab (Fig. 8).

Experimental procedures

Animals and primary cultured hemocytes

Our experiments followed the protocol approved by the East China Normal University Animal Care and Use Committee (protocol license number AR2012/12017) in direct accordance with the Ministry of Science and Technology (China) for animal care guidelines. Healthy *E. sinensis* crabs (100 ± 10 g each for adults and 15 ± 2 g each for naïve crabs, nonantibiotic or antifungal fed) were obtained from the Songjiang aquatic farm (Shanghai, China). After quickly transferring them to the Biological Experiment Station at East China Normal University, all crabs were maintained in filtered and aerated freshwater with plenty of oxygen provided and fed daily a commercial formulated diet containing no antibiotics.
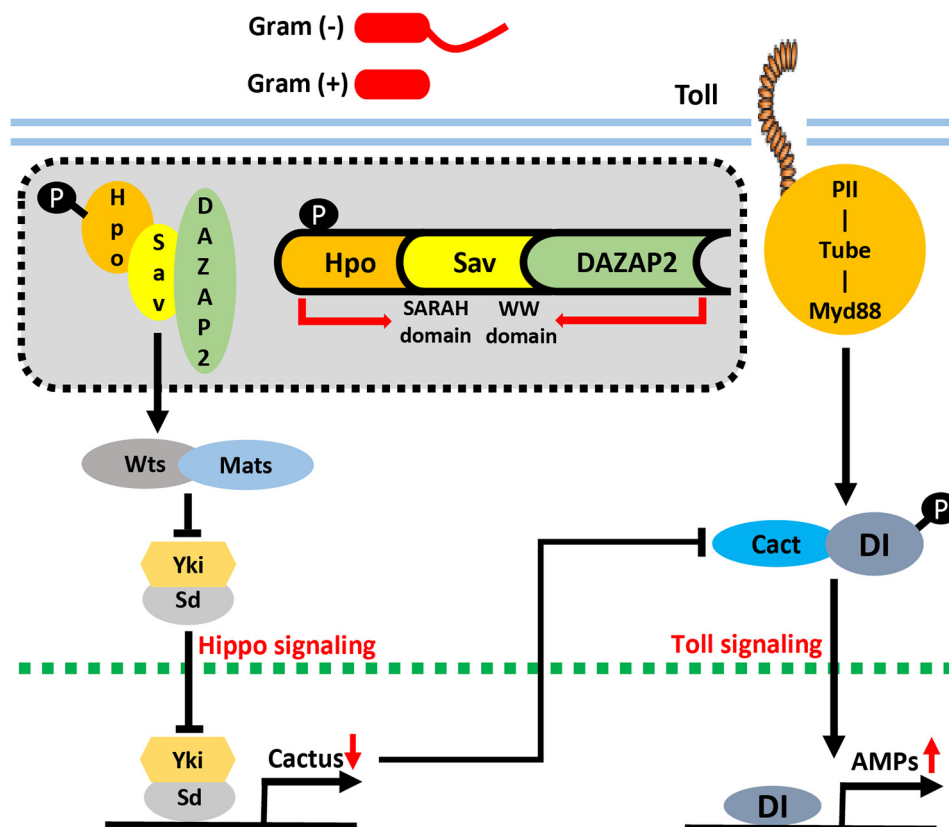
Figure 8. Schematic representation of DAZAP2-regulated antimicrobial activities in crab. Bacteria induced high levels of DAZAP2 expression in crab, with DAZAP2 subsequently binding to Sav, a core molecule in Hippo signaling, via the association of DAZAP2 and WW domains. This functioned to enhance the phosphorylation of Hpo, which in turn can also bind to Sav via the SARAH domain. Because 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.

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

Immune stimulation and sample collection

S. aureus and *V. 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 resuspended 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 the stimulation. Three or more crabs were used per sample. First-strand cDNA was synthesized using a reverse transcriptase kit (Takara, Osaka, Japan), 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 nonsclerotized 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*) and then sent to the Novogene Co. (Beijing, China) for whole-genome sequencing on Illumina HiSeq 2000 and PacBio platforms. Among this genomic data, we identified a fragment that contained a full ORF 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 version 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/)[5] (36) and SMART (http://smart.embl-heidelberg.de/).[5] An unrooted phylogenic tree was constructed based on the deduced amino acid sequences of *Es*DAZAP2 and other known DAZAP2 sequences by the neigh-

[5] Please note that the JBC is not responsible for the long-term archiving and maintenance of this site or any other third party hosted site.

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bor-joining algorithm using MEGA version 6.0 software. To derive a confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

RNAi 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 S1), 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 Co. (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 Scientific) with a final concentration of 10 nM. For the *in vivo* RNAi, the siRNA (1 µg/g) was injected into crab hemolymph (of the nonsclerotized membrane of the posterior walking leg). To extend the RNAi effect, 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* was 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 S1), by using CFX96^{TM} Real-Time System (Bio-Rad) 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 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 ± S.D.

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 was 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 blotting

Protein samples were obtained from hemocytes by using the Coomassie Plus protein assay reagent (Thermo Fisher Scientific, Waltham, MA). Whole-cell lysates were obtained by lysing cells with a RIPA buffer containing a mixture of protease and phosphatase inhibitors (Roche Applied Science), with protein concentration quantified by a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Next, the protein was separated by 10% SDS-PAGE, transferred onto a nitrocel-

lulose membrane, and blocked for 1–2 h with 3% nonfat milk in 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 nonspecific 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 and then incubated 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, MA). 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, NE).

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, and 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 and then washed with PBS. Hemocytes were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, AnaSpec Inc., San Jose, CA), for 10 min at room temperature, and then washed again. All stained hemocytes were then observed under a Revolve Hybrid Microscope (Echo).

Yeast two-hybrid library screening

The cDNA library for Y2H experiments was built by OE Bio-Tech (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 S1) 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^{TM} 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) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). 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 Scientific, Waltham, MA) 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 RIPA buffer, followed by centrifugation at $14,000 \times g$ 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 $12,000 \times g$ 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, washed with PBS, and then resuspended in the SDS-PAGE loading buffer for separation by SDS-PAGE and 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, NE).

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

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