An inhibitor of apoptosis protein (EsIAP1) from Chinese mitten crab *Eriocheir sinensis* regulates apoptosis through inhibiting the activity of EsCaspase-3/7-1

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Inhibitor of apoptosis proteins (IAPs) maintain the balance between cell proliferation and cell death by inhibiting caspase activities and mediating immune responses. In the present study, a homolog of IAP (designated as *Es*IAP1) was identified from Chinese mitten crab *Eriocheir sinensis*. *Es*IAP1 consisted of 451 amino acids containing two baculoviral IAP repeat (BIR) domains with the conserved Cx2 Cx6 Wx3 Dx5 Hx6 C motifs. *Es*IAP1 mRNA was expressed in various tissues and its expression level in hemocytes increased significantly (*p* < 0.01) at 12–48 h after lipopolysaccharide stimulation. In the hemocytes, *Es*IAP1 protein was mainly distributed in the cytoplasm. The hydrolytic activity of recombinant *Es*Caspase-3/7-1 against the substrate Ac-DEVD-pNA decreased after incubation with r*Es*IAP1. Moreover, r*Es*IAP1 could directly combine with r*Es*Caspase-3/7-1 in vitro. After *Es*IAP1 was interfered by dsRNA, the mRNA expression and the hydrolytic activity of *Es*Caspase-3/7-1 increased significantly, which was 2.26-fold (*p* < 0.05) and 1.71-fold (*p* < 0.05) compared to that in the dsGFP group, respectively. These results collectively demonstrated that *Es*IAP1 might play an important role in apoptosis pathway by regulating the activity of *Es*Caspase-3/7-1 in *E. sinensis*.

Apoptosis is a type of cell death which plays an important role in regulating growth, development, and immune responses⁵,⁶. Apoptosis is tightly controlled by multiple regulators, and the interaction between positive and negative regulators determines whether this program is activated or inhibited⁷. A family of cysteine-aspartic specific proteases known as caspases are considered as the executors of apoptosis, which cleave their substrates after the aspartate residue leading to protein degradation and apoptosis⁸. The modulation of apoptosis can be achieved by the dynamic expression of BCL-2 protein family members as well as the inhibitor of apoptosis proteins (IAPs)⁵,⁶.

IAP was firstly recognized in baculoviruses which could inhibit apoptosis in infected cells in 1993⁷. Subsequently, numerous IAP homologues have been identified in vertebrates, which is primarily divided into five groups including X-linked IAP (XIAP), c-IAP1, c-IAP2, NAIP, and Survivin⁹. All the IAPs contain one to three baculovirus IAP repeats (BIR) domains, which is consisted of approximately 70 amino acid residues⁹. The IAP family members differ in the number of BIR domains, and some of them also contain a RING finger domain. XIAP, c-IAP1 and c-IAP2 comprise three BIRs in the N-terminus and a RING finger in the C-terminus, while NAIP contains three BIRs without RING finger domain, and Survivin and BRUCE include only one BIR⁹. Accumulating evidences have favored that some vertebrate IAPs, such as XIAP, c-IAP1 and c-IAP2, could directly bind to the activated caspase-3 and -7, and inhibit their activities⁸,¹⁰. The BIR domains have been suggested to be responsible for the inhibition of caspases¹⁰,¹¹. For instance, the BIR motifs of c-IAP1 and c-IAP2 from *Homo sapiens* were evidenced for their ability to inhibit active recombinant caspases in vitro¹⁰. The BIR2 of XIAP from *H. sapiens* were evidenced for their ability to inhibit active recombinant caspases in vitro¹⁰. The BIR2 of XIAP from *H. sapiens* were evidenced for their ability to inhibit active recombinant caspases in vitro¹⁰. The BIR2 of XIAP from *H. sapiens* were evidenced for their ability to inhibit active recombinant caspases in vitro¹⁰.

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sapiens, but not the BIR1 or the BIR3, was able to interact with caspase-3 and -7 with an apparent inhibition. It was reported that the RING finger domain in IAPs could coordinate two zinc atoms. In H. sapiens, the RING finger domain of XIAP could act as an E3 ubiquitin ligase. Moreover, it could also recruit E2 ubiquitin-conjugating enzymes and transfer ubiquitin to its target proteins bound to IAPs.

Recently, IAP homologues have also been discovered in various species of invertebrates. The invertebrate IAP homologues also contain one to three BIR domains, and some of them possess a RING finger domain. Some invertebrate IAPs were found to share the similar function with their homologues in vertebrates, which could play vital roles in the regulation of apoptosis and immune response against invading pathogens. For example, DIAP1 and DIAP2 were identified in fruit fly Drosophila melanogaster with two and three BIR domains, respectively. DIAP2 was able to regulate the expression of antimicrobial peptides (AMPs) in response to gram-negative bacterial infection through modulating the immune deficiency (IMD) pathway in D. melanogaster. LvIAP1 with three BIR domains identified from shrimp Litopenaeus vannamei was reported to play vital roles in the regulation of shrimp hemocyte apoptosis response against white spot syndrome virus stimulation. Two IAPs (named as CgIAP1 and CgIAP2) containing two BIR domains were characterized in Pacific oyster Crassostrea gigas, and they were found to be involved in regulating the apoptosis pathway and immune defense against bacterial infections. It has been demonstrated that some IAPs could inhibit the activation of caspases in invertebrates. DIAP1 could interact with caspase DRONC and interfere its activation in D. melanogaster. The BIR2 domain of CgIAP2 could interact with the initiator caspase CgCaspase-2 to participate in apoptosis inhibition. The diverse caspase family members have also been discovered in various species of crustacean. For example, three caspases (EsCaspase-3, -7 and -8) were characterized in E. sinensis to play crucial roles in Cadmium-induced apoptosis, and two caspases (EsCaspase-3/7-1 and EsCaspase-3-like) were involved in innate immune response under pathogen induced apoptosis. In shrimp L. vannamei, there were four caspases (LvCaspase-2, -3, -4 and -5) identified to play role in the host defense against white spot syndrome virus. Compared with vertebrate IAPs and caspases, the knowledge about the interaction modes of the large family of invertebrate IAPs and caspases as well as their involvements in apoptosis is still quite meagre.

E. sinensis is one of the important economic species, and the industry of E. sinensis aquaculture has been increasing rapidly in China. With the development of aquaculture, various diseases caused by bacteria, viruses or other pathogenic organisms have frequently occurred in cultured E. sinensis and caused catastrophic losses. Therefore, the better understanding of immune response mechanism is helpful for controlling the diseases and reducing economic losses. In crabs, hemocytes are found to play crucial roles in defending against pathogen invasion and they can be induced to apoptosis after pathogen stimulation. IAPs as inhibitors of apoptosis proteins play critical roles in inhibiting the cell apoptosis. In the present study, a novel IAP (designated as EsiIAP1) was identified from Chinese mitten crab E. sinensis with the objectives (1) to investigate its mRNA distribution and its mRNA expression profile in response to immune stimulations, (2) to determine its subcellular localization, (3) to validate the interaction of rEsiIAP1 and rEsCaspase-3/7-1 in vitro, as well as the potential regulation between EsiIAP1 and EsCaspase-3/7-1 in vivo, hopefully to provide more information to understand the apoptosis regulation mechanism in crustaceans.

Results
The sequence characteristics and phylogeny of EsiIAP1. A novel sequence of EsiIAP1 (GenBank accession numbers MF351747) was identified from E. sinensis genome database. The open reading frame of EsiIAP1 was of 1,356 bp, encoding a predicted polypeptide of 451 amino acids with calculated molecular weight of approximately 50 kDa. SMART analysis demonstrated that EsiIAP1 contained two BIR domains (BIR1 and BIR2). The conserved cysteine and histidine residues and the spacing between them in the reported BIR2 domains (Cx, Cx, Wx, Dx, Hx, C) were also identified in the BIR2 domain of EsiIAP1 (Fig. 1a). The deduced amino acid sequences of BIR1 and BIR2 domains of EsiIAP1 shared high sequence similarities with the corresponding domains of other IAPs, such as those from L. vannamei IAP1 (40.5% and 50.7%), Mus musculus XIAP (43.1% and 45.9%), H. sapiens c-IAP2 (45.8% and 53.4%), H. sapiens XIAP (41.7% and 47.3%), M. musculus c-IAP2 (44.4% and 49.3%), D. melanogaster DIAP2 (41.7% and 46.6%), Bombyx mori IAP (53.5% and 58.9%), H. sapiens c-IAP1 (43.1% and 50.7%), M. musculus c-IAP (43.1% and 50.7%), C. gigas (36.1% and 57.5%), and Penaecus monodon IAP (40.5% and 50.7%) (Fig. 1b). To evaluate the evolutionary relationship of EsiIAP1, a phylogenetic tree was constructed based on the amino acid sequences of 13 IAP members by the neighbor-joining method. EsiIAP1 was firstly clustered with other arthropod IAPs in the phylogenetic tree, and then grouped with invertebrate IAPs, and finally clustered into the vertebrate XIAPs and c-IAPs (Fig. 1c).

Tissue distribution of EsiIAP1 mRNA and subcellular localization of EsiIAP1 in hemocytes. The mRNA transcripts of EsiIAP1 could be detected in all the examined tissues, including hemocytes, hepatopancreas, heart, gill, brain and muscle with the highest expression level in hepatopancreas, which was 5.24-fold (p < 0.01) of that in muscle. The expression level of EsiIAP1 mRNA in gill, hemocytes, heart and brain was 3.51-fold (p < 0.01), 3.41-fold (p < 0.01), 2.16-fold (p < 0.05) and 1.61-fold (p > 0.05) of that in muscle, respectively (Fig. 2a).

The purified rEsiIAP1 was employed to prepare polyclonal antibody (Fig. 2b, Supplementary Fig. S1). A clear band with 55 kDa was revealed by western blotting assay, indicating the high recognition specificity of the polyclonal antibody against EsiIAP1 (Fig. 2b, Supplementary Fig. S1). Pre-immune serum was used as negative control and no bands were detected (Fig. 2b, Supplementary Fig. S1). Western blotting assay of the native tissue sample with EsiIAP1 antibody revealed that there was a distinct band of 50 kDa (Fig. 2c). Immunofluorescence assay was performed to detect the localization of EsiIAP1 in hemocytes. The nucleus stained by DAPI was observed in blue, and the positive signal of EsiIAP1 was in green. The positive fluorescence signals were mainly observed in the cytoplasm of hemocytes according to the merged pictures (Fig. 2d).
The expression of EsiIAP1 mRNA in hemocytes after LPS and A. hydrophila stimulations. The expression level of EsiIAP1 mRNA in hemocytes increased significantly after the stimulations with LPS and A. hydrophila. After LPS stimulation, the mRNA transcripts of EsiIAP1 increased significantly at 12 h (2.80-fold
compared with that in PBS group, $p < 0.01$), reached the highest level of 13.86-fold ($p < 0.01$) at 24 h, and finally down-regulated to 2.44-fold ($p < 0.05$) (Fig. 3a). After A. hydrophila stimulation, the relative expression level of EsIAP1 mRNA kept at quite low level and there was no significant difference from 0 to 24 h compared with that in PBS group. However, it increased significantly (2.71-fold of control group, $p < 0.01$) at 48 h post A. hydrophila stimulation (Fig. 3b).

The mRNA expression of EsCaspase-3/7-1 and the activity of caspases in hemocytes after the gene silencing of EsIAP1. To further explore the function of EsIAP1 in vivo, the dsRNA-induced RNA interference (RNAi) was used to inhibit the expression of EsIAP1. The mRNA expression of EsIAP1 in hemocytes decreased to 0.47-fold ($p < 0.05$) (Fig. 4a), while the mRNA expression of EsCaspase-3/7-1 increased to 2.26-fold ($p < 0.05$) (Fig. 4b) at 24 h post the injection with sequence-specific dsRNA targeting EsIAP1 compared to that in dsGFP group. After EsIAP1 was silenced, the activity of caspase towards Ac-DEVD-pNA in hemocytes increased to 1.71-fold ($p < 0.05$) compared to that in the dsGFP group. While the activity of caspase-1 towards Ac-YAVD-pNA and caspase-6 towards Ac-VEID-pNA in hemocytes increased to 1.21-fold ($p < 0.05$) and 1.25-fold ($p < 0.05$) of that in the dsGFP group, respectively (Fig. 4c).

The interaction of rEsIAP1 with rEsCaspase-3/7-1 in vitro. The interaction of EsIAP1 with EsCaspase-3/7-1 was analyzed by pull down assay to understand the regulatory mechanism of apoptosis. The full-length ORFs of EsIAP1 and EsCaspase-3/7-1 were expressed, and the purified rEsIAP1 and rEsCaspase-3/7-1 (Fig. 5a-b, Supplementary Fig. S2a-b) were used for GST and His pull down assays. Two distinct bands were observed in the elute liquid after pull down assay (Fig. 5c-d, Supplementary Fig. S2c-d). The results indicated that rEsIAP1 could directly combine and interact with rEsCaspase-3/7-1 in vitro.

The hydrolytic activity of rEsCaspase-3/7-1 after incubation with rEsIAP1 in vitro. The hydrolyzing assay of caspase-3 substrate was employed to investigate the activity of rEsIAP1. The hydrolytic activity of rEsCaspase-3/7-1 was significantly inhibited after the incubation with rEsIAP1. rEsCaspase-3/7-1 displayed high hydrolytic activity towards Ac-DEVD-pNA (0.44 units per mg protein). After rEsCaspase-3/7-1 was incubated with rEsIAP1 or Z-VAD-FMK, the hydrolytic activities were 0.25 and 0.15 units per mg protein, respectively, which were significantly lower than that in rEsCaspase-3/7-1 group ($p < 0.05$) (Fig. 5e).

Discussion
Apoptosis represents a fundamental biological process that relies on the activation of caspases. IAPs are a family of negative regulators of both caspases and cell death. In the present study, a novel IAP was identified from E. sinensis (designated EsIAP1). There were two BIR domains identified in EsIAP1, which was the typical domain of IAP family. The deduced amino acid sequences of BIR1 and BIR2 domains in EsIAP1 shared high similarities (36.1%–53.5% and 45.9%–58.9%, respectively) with the corresponding domains of other IAP proteins (Fig. 1a,b). Moreover, the conserved spacing of cysteine and histidine residues (Cx2 Cx6 Wx3 Dx5 Hx6 C) in the other reported BIR2 domains were also found in EsIAP1, which was suggested to contribute to a novel zinc-binding fold. These results suggested that EsIAP1 was a typical IAP family member. In the phylogenetic tree, EsIAP1 was firstly grouped with the crustacean IAPs to form a separated clade, then grouped with other arthropod IAP proteins, and finally clustered with vertebrate IAP proteins (Fig. 1c). These evidences collectively indicated that EsIAP1 belonged to the IAP family in crustaceans.

As regulators of the apoptotic machinery, IAPs play important roles in many physiological processes, including homeostasis maintenance, development of tissues, and immune responses. In the present study, the mRNA transcripts of EsIAP1 could be detected in all the examined tissues including hemocytes, hepatopancreas, gill, muscle, brain, and heart (Fig. 2a). Similarly, the transcripts of CgIAP2 were also detected in various tissues in oyster C. gigas. The constitutive expression profile of EsIAP1 indicated that it might involve in...
Figure 4. The mRNA and activity of caspase after the gene silencing of EsIAP1. (a) The expression level of EsIAP1 mRNA in hemocytes after gene silencing of EsIAP1. Comparison of the level of EsIAP1 was normalized to dsGFP group. (b) The expression level of the EsCaspase-3/7-1 mRNA in hemocytes of EsIAP1-interfered crabs. Comparison of the level of EsIAP1 was normalized to dsGFP group. (c) The activities of caspases were determined by measuring hydrolyzing activity against Ac-YVAD-pNA (substrate of caspase-1), Ac-DEVD-pNA (substrate of caspase-3) or Ac-VEID-pNA (substrate of caspase-6).

many physiological processes of crabs. It has been reported that IAPs could regulate the activity of caspases, further modulate cell cycle proliferation and receptor-mediated signal transduction8. The higher level of EsIAP1 mRNA was observed in immune-associated tissues, including hemocytes, hepatopancreas and gill, which might be attributed to the cellular metabolism and innate immunity38,39. Crustacean hemocytes play important roles in the host immune response, including recognition, phagocytosis and cell communication33,40,41. Moreover, the high level of EsCaspase-3/7-1 and EsCaspase-3-like were observed in hemocytes28,29. The hemocytes were thus chosen as target to analyze the expression of EsIAP1. In the present study, the location of EsIAP1 in hemocytes was observed by immunofluorescence assay, and the positive signal was found to be mainly distributed in the cytoplasm, which was similar as the previous reports in other species42,44, possibly for the sake of binding to cytoplasmic caspase to regulate hemocyte apoptosis. LPS, a vital component of the outer wall of gram-negative bacteria, could trigger caspase-mediated apoptosis pathway42,46. The apoptosis pathway is regulated by initiator caspases (such as aspase-8 and caspase-10), which can be triggered by death receptor (like Fas, TNFR1 and TRAIL-R1/R2) to initiate the activity of effector caspases43–45. In the present study, the expression level of EsIAP1 mRNA was significantly up-regulated after LPS and A. hydrophila stimulations (Fig. 3a,b). It has been reported that apoptosis pathway could be activated after LPS and A. hydrophila stimulations in crustaceans37,43. In C. gigas, CgIAP2 was proposed to play a role in apoptosis inhibition in the immune defense against bacterial challenge37. Some crustacean IAPs such as PmIAP and LvIAP1 were suggested to be central to the regulation of hemocyte apoptosis4,49. These results suggested that EsIAP1 might exert important roles in immune defenses by regulating the apoptosis pathway in E. sinensis.

IAPs could regulate apoptosis through controlling caspase activities and caspase-activating platform formations, which also appeared to be important determinants of the responses of cells to endogenous or exogenous cellular injuries41. It was reported that c-IAP1 and c-IAP2 could directly bind to the activated caspase-3 and -7 to inhibit their activities in vertebrates40. In the present study, EsCaspase-3/7-1, an effector caspase, identified previously from E. sinensis48, were employed to investigate the binding activity of EsIAP1 with caspase. After rEsCaspase-3/7-1 was incubated with rEsIAP1 or Z-VAD-FMK, the hydrolyzing activity of rEsCaspase-3/7-1 was significantly decreased (Fig. 5e). This result was in coincidence with the observation that IAPs could inhibit the activation of effector caspase3,48. The direct combination of rEsIAP1 with rEsCaspase-3/7-1 confirmed by pull down assay might explain the decrease of rEsCaspase-3/7-1 hydrolyzing activity after incubation with rEsIAP1 in vitro. These results suggested that rEsIAP1 could inhibit the hydrolytic activity of rEsCaspase-3/7-1 by interacting with rEsCaspase-3/7-1. Furthermore, the expression of EsCaspase-3/7-1 mRNA in hemocytes of crabs were significantly increased after the interference of EsIAP1, indicating the inhibitory regulation of EsIAP1 on EsCaspase-3/7-1. The hydrolytic activity of caspase-3 was increased in hemocytes rather than caspase-1 and -6 after EsIAP1 was silenced. These results showed that EsIAP1 could regulate EsCaspase-3/7-1 and further inhibit hemocyte apoptosis in vivo. Similarly, the number of circulating hemocytes was increased in LvIAP1-silenced shrimp because of the extensive apoptosis44. Some mammalian IAPs, such as c-IAP1 and c-IAP2, were also found to be involved in signaling cascades, and play important roles in TNF-induced apoptosis9. Therefore, it was speculated that EsIAP1 could inhibit apoptosis by regulating EsCaspase-3/7-1 in E. sinensis.

Caspases are activated to gain the full catalytic activity after being proteolytically cleaved to initiate apoptosis51. EsCaspase-3, -7 and -8 are characterized to play crucial roles in Cadmium-induced apoptosis52, and EsCaspase-3/7-1 and EsCaspase-3-like protein are found to be involved in innate immune response and induce apoptosis under pathogen stimulation28,29. IAPs inhibit such proteolytically activated caspases, and further regulate apoptosis52. The loss or inhibition of cIAP1, cIAP2 and XIAP causes the majority of cells to be sensitized to death receptor to induce cell death53. In Drosophila, DIA1 normally inhibits both initiator and effector caspases54,55. In summary, this study suggested that LPS and bacterial challenge could activate the apoptosis pathway in E. sinensis. EsIAP1 could inhibit apoptosis by directly combining with EsCaspase-3/7-1 and inhibit
its hydrolytic activity (Fig. 5f). These results provided novel idea to understand the modulatory role of IAP in apoptosis pathway in crustaceans.

Materials and Methods

**Crabs, collection of tissues and immune stimulations.** The crabs with an average weight of 20 g were collected from a commercial farm in Lianyungang, China, and cultured in aerated freshwater at 20 ± 2 °C for one week before processing29. Six crabs were sacrificed for determining the expression of EsIAP1 mRNA in different tissues. The tissues including muscle, heart, brain, hepatopancreas and gill were collected from crabs to detect the mRNA expression of EsIAP1 according to the previous study56. The hemolymph drawing from the last pair of walking legs from each crab by using a syringe was mixed with anticoagulant solution (510 mM NaCl, 100 mM glucose, 200 mM citric acid, 30 mM sodium citrate, 10 mM EDTA·2Na, pH 7.3) at a ratio of 1:1, and the hemocytes were harvested by centrifugation57. Tissues from two crabs were pooled together as one sample and there were three duplicates for each assay according to the previous methods58. The crabs were treated by the injections
of 100 μL *Aeromonas hydrophila* (10^7 CFU mL^-1^) and 100 μL lipopolysaccharide (500 μg mL^-1^) according to the previous reports^59^, respectively. Ninety crabs were employed and randomly divided into three groups. According to previous study, a volume of 100 μL alive *A. hydrophila* (1 x 10^7 CFU mL^-1^) or lipopolysaccharide (LPS from *Escherichia coli* 0111:B4, L2630, Sigma Aldrich, USA; 100 μg mL^-1^) resuspended in PBS (40 mM NaCl, 2.7 mM KCl, 10 mM NaHPO4, 2 mM KH2PO4, pH 7.4) was injected into the arthrodial membrane of the last pair of walking legs in the stimulation groups, respectively^28,59,60^. The crabs received an injection of 100 μL PBS were employed as control group. Six crabs were randomly sampled from each group at 0, 6, 12, 24 and 48 h after treatments.

**RNA extraction and cDNA synthesis.** TRIzol reagent (Invitrogen) was used for the extraction of total RNA from *E. sinensis* tissue samples, and the first-strand cDNA was synthesised by using the PrimeScript™ real-time PCR kit (Takara, Japan) according to the manufacturer’s instruction.

**Sequence analysis of EsIAP1.** The sequence of IAP genes was analyzed by BLASTP (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the genome database (PRJNA305216) of *E. sinensis*^61^. The primers (EsIAP1-F and -R) were designed to clone the open reading frame (ORF) of EsIAP1. The multiple sequence alignments were created by Clustal W. The conserved domain was identified through the SMART (http://smart.embl-heidelberg.de/). MEGA6.0 package was used to construct phylogenetic tree.

**Purification of recombinant protein and preparation of polyclonal antibody.** The full-length ORF sequences of EsIAP1 and EsCaspase-3/7-1 were amplified with specific primers (tEsIAP1-His-F and -R, rEsCaspase-3/7-1-F and -R) (Table 1). The PCR products were inserted into pET-22b vector (Novagen) with a His-tag. rEsIAP1-GST-F and -R (Table 1) were used to amplify EsIAP1, and the PCR products were inserted into the pGEX4T-1 vector (GE Healthcare) with a GST-tag. All those recombinant plasmids were transformed into *Escherichia coli* BL21 (DE3) competent cells. These prokaryotic proteins were purified by a Ni2+ chelating sepharose column or GST-resin, following the manufacturers' instructions. Their concentrations were measured by BCA kit (Pierce). The RNA interference assay of *E. sinensis* was performed according to the previous report^32^. Recombinant protein was separated by SDS-PAGE, and transferred onto nitrocellulose membrane. After blocking for 1 h with 5% non-fat milk in TBST, the membrane was incubated successively with 1/1000 diluted poly-antibody of anti-EsIAP1 as first antibody and 1/10,000 diluted goat-anti-mouse (Sigma Aldrich, USA) as secondary antibody. Then the membrane was incubated for 2 min with ECL substrate system (Thermo Scientific) for 2 min, then imaged by Amersham Imager 600 (General Electric Company).

**Western blotting and immunohistochemistry analysis of EsIAP1.** The western blotting assay was performed according to the previous report^46^. Recombinant protein was transferred onto nitrocellulose membrane. After blocking for 1 h with 5% non-fat milk in TBST, the membrane was incubated successively with 1/1000 diluted poly-antibody of anti-EsIAP1 as first antibody and 1/10,000 diluted goat-anti-mouse IgG (Sangon) as secondary antibody. After washing in TBST, the membrane dipped in ECL substrate system (Thermo Scientific) for 2 min, then imaged by Amersham Imager 600 (General Electric Company).

**RNA interference.** The RNA interference assay of EsIAP1 was performed according to the previous report^42^. T7 promoter linked primers (GFP-RNAi-F and -R, EsIAP1-RNAi-F and -R) were used to amplify the cDNA sequence of dsGFP (657 bp) and dsEsIAP1 (1,356 bp), respectively. The dsRNAs of EsIAP1 and GFP were diluted

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| Primers | Sequence (5′-3′) |
|---------|----------------|
| EsIAP1-F | ATGGACATGTCCTCGTCGCGCCAGTT |
| EsIAP1-R | TCAGCCGATGAGTGGGCCG |
| rEsCaspase-3/7-1-F | GGGAATTCCATATAGGAGGACATGTCGCGCGCCAGTT |
| rEsCaspase-3/7-1-R | CGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| rEsIAP1-F (His) | GGAAATTCATATAGGAGGACATGTCGCGCGCCAGTT |
| rEsIAP1-R (His) | GGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| rEsIAP1-F (GST) | CGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| rEsIAP1-R (GST) | CGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| EsIAP1-RNAi-F | TAATACGACCTACTATAGGAGGACATGTCGCGCGCCAGTT |
| EsIAP1-RNAi-R | TAATACGACCTACTATAGGAGGACATGTCGCGCGCCAGTT |
| GFP-RNAi-F | TAATACGACCTACTATAGGAGGACATGTCGCGCGCCAGTT |
| GFP-RNAi-R | TAATACGACCTACTATAGGAGGACATGTCGCGCGCCAGTT |
| EsIAP1-qRT-F | CGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| EsIAP1-qRT-R | CATCAAGGAGAAACTGTGCT |
| EsIAP1-F (GST) | CGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| EsIAP1-R (GST) | CGCGGATCCATATAGGAGGACATGTCGCGCGCCAGTT |
| Caspase-3/7-1-qRT-F | CCACCACTGCTGTCATCAAA |
| Caspase-3/7-1-qRT-R | AGACAGGAAGACCTTTCTCATCAAA |
| β-Actin-F | CCCATCTACGAGGGCTACGC |
| β-Actin-R | CCTTGATGTCTCGCACGAC |

Table 1. Primers used in this study.
with PBS to the final concentration of 0.5 μg mL⁻¹. The crabs were treated by the injections with 100 μL PBS, dsGFP and dsEsIAP1, respectively. The untreated crabs were used as blank group. Six crabs from each group were randomly sampled at 0 and 24 h post injections. The hemocytes were divided into two parts, and one aliquot of hemocyte sample was used to estimate the silencing efficiency, while the other was used for the measurement of caspase activity.

**Quantitative real-time PCR (qRT-PCR) analysis of mRNA expression.** qRT-PCR was conducted according the previous reports. Two primers, EsIAP1-qRT-F and -R, were used in qRT-PCR to detect the expression of EsIAP1. The fragment amplified by primers of Es-β-actin (Es-β-Actin-F and -R) were employed as reference. The gene expression analysis was performed using the 2⁻ΔΔCt method, and all data were given in terms of relative mRNA expression of mean ± S.E. (N = 3).

**Pull down assay.** The pull down assay was carried out according to the previous report. The proteins of rEsIAP1 (GST) and rEscaspass-3/7-1 (His) (30 μg) were mixed with 20 μL of glutathione resin (for GST-tagged proteins) or charged nickel-nitritoltriacetic acid beads (for His-tagged proteins), respectively. The mixture (resin and binding proteins) was incubated at room temperature for 2 h with slight rotation, and then washed for three times by centrifuging at 500 × g for 3 min to remove the unbound proteins. The tested protein (rEscaspass-3/7-1-His and rEsIAP1-GST), without GST tag or His tag, was added into the mixture containing the nickel-nitritoltriacetic acid beads or glutathione resin, and gently rotated at room temperature for 2 h. After washing three times, the mixture was analyzed by SDS-PAGE.

**The hydrolyzing function assays of rEsIAP1 in vitro.** The potential inhibitory hydrolytic activity of rEsIAP1 was detected using the caspase-3 activity kit (Beyotime) under the manufacturer’s manual. The protein concentration of purified rEsIAP1-His and rEscaspass-3/7-1 was adjusted to 1 mg mL⁻¹. There were three experimental groups, including blank group (rEscaspass-3/7-1), rEsIAP1 group (rEscaspass-3/7-1 + EsIAP1), and Z-VAD-FMK (pan caspase inhibitor) group (Z-VAD-FMK + rEscaspass-3/7-1). The mixtures were incubated at 37 °C for 1 h and absorbance value was monitored at 405 nm by the SpectraMax 190 (Molecular Devices, Sunnyvale, CA, USA). The blank group (rEscaspass-3/7-1) was employed as the control, and the hydrolytic activity of rEsIAP1 was determined by comparing the hydrolytic activity of rEscaspass-3/7-1 against Ac-DEVD-pNA.

**Hydrolyzing activity analysis of caspases in EsIAP1-interfered crabs.** The hydrolyzing activity of caspases in hemocytes was examined according to the method described by previous study. The hydrolytic activity of the crab hemocyte protein was detected at 0 and 24 h after the injection of EsIAP1 dsRNA. The protein concentration of the supernatant was measured using the Bradford Protein Assay Kit (Beyotime) and adjusted to 1 mg mL⁻¹ with lyase buffer. The hydrolytic activity of caspases was examined with the substrate Ac-YAVD-pNA, Ac-DEVDPNA and Ac-VEID-pNA using the caspase-1, -3 and -6 activity assay kit (Beyotime, Shanghai, China) under the manufacturer’s manual. The absorbance values of the reaction mixture was monitored at 405 nm using Spectra Max 190 (Molecular Devices, Sunnyvale, CA, USA). The absorbance values represented the cleavage and release of pNA. The blank group was used as the reference.

**Statistical analysis.** The data (represented as mean ± S.E., N = 3) were calculated by using the 2⁻ΔΔCt method, and analyzed with t-test. Significant differences across controls were indicated with an asterisk at p < 0.05, and two asterisks at p < 0.01.

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**Author contributions**
C.Q. prepared the experiments and figures. Experiments were assisted by J.-J.S., Q.-S. X., X.-J.L., W.Y., F.-F.W., Y.W., Q.-L.Y., Z.-H.J., L.-L.W. and L.-S.S. supervised the work. The manuscript was written by C.Q., J.-J.S. and edited by L.-S.S.

**Competing interests**
The authors declare no competing interests.

**Additional information**
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