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## $\beta$ -Arrestin 1's Interaction with TC45 Attenuates Stat signaling by dephosphorylating Stat to inhibit antimicrobial peptide expression

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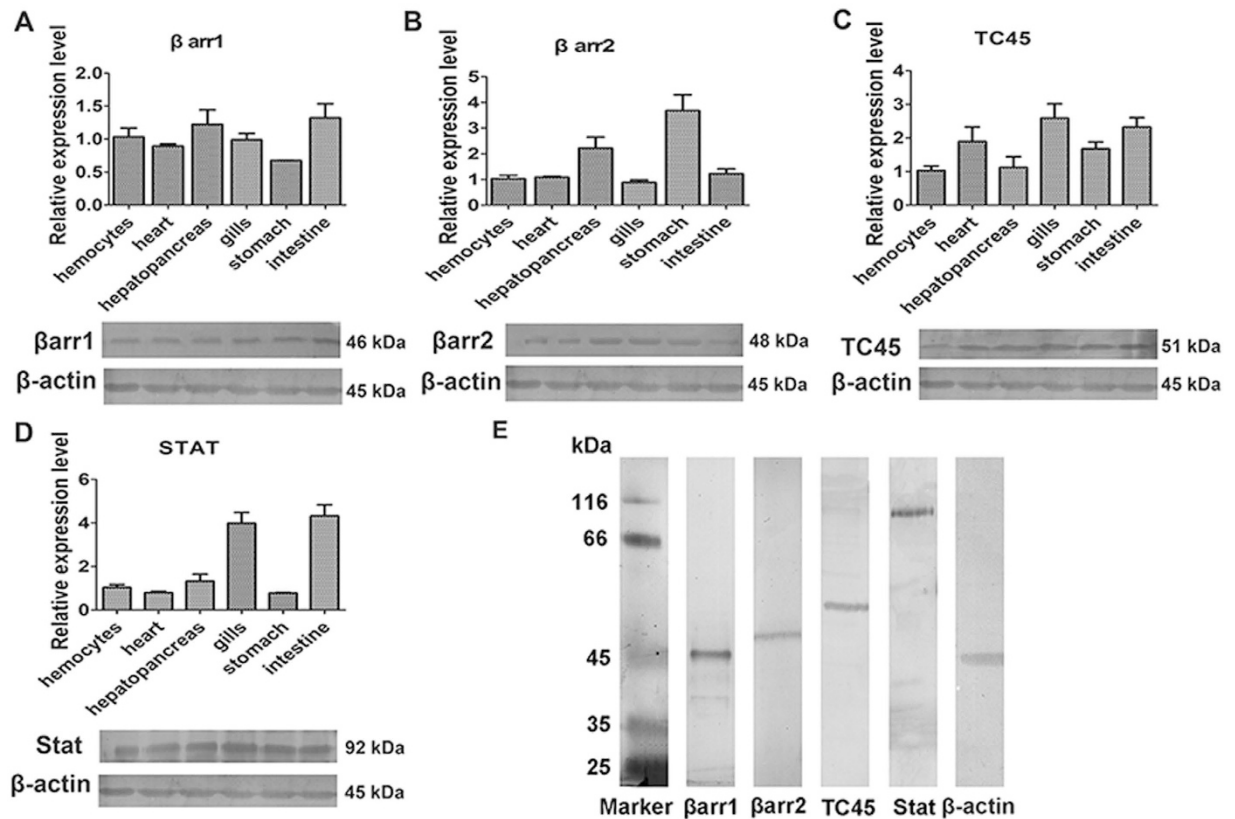
Impaired phosphatase activity leads to the persistent activation of signal transducers and activators of transcription (Stat). In mammals, Stat family members are often phosphorylated or dephosphorylated by the same enzymes. To date, only one Stat similar to mammalian Stat5a/b has been found in crustaceans and there have been few studies in Stat signal regulation in crustaceans. Here, we report that  $\beta$ -arrestin1 interacts with TC45 (45-kDa form of T cell protein tyrosine phosphatase) in the nucleus to attenuate Stat signaling by promoting dephosphorylation of Stat. Initially, we showed that Stat translocates into the nucleus to induce antimicrobial peptide (AMP) expression after bacterial infection.  $\beta$ Arr1 enters the nucleus of hemocytes and recruits TC45 to form the  $\beta$ arr1-TC45-Stat complex, which dephosphorylates Stat efficiently. The interaction of TC45 with Stat decreased and Stat phosphorylation increased in  $\beta$ arr1-silenced shrimp (*Marsupenaeus japonicus*) after challenge with *Vibrio anguillarum*.  $\beta$ Arr1 directly interacts with Stat in nucleus and accelerates Stat dephosphorylation by recruiting TC45 after *V. anguillarum* challenge. Further study showed that  $\beta$ arr1 and TC45 also affect AMP expression, which is regulated by Stat. Therefore,  $\beta$ arr1 and TC45 are involved in the anti-*V. anguillarum* immune response by regulating Stat activity negatively to decrease AMP expression in shrimp.

The Janus kinase (Jak)/signal transducers and activators of transcription (Stat) signaling pathway participates in cell proliferation, differentiation, development, survival and apoptosis<sup>1–3</sup>. Mammals, have Stats (Stat1, Stat2, Stat3, Stat4, Stat5a, Stat5b, Stat6), which are activated by tyrosine phosphorylation and translocated into the nucleus, where they bind to promoters to initiate transcription of effector genes<sup>4,5</sup>. Activators of Stats include epidermal growth factor receptor (EGFR)<sup>6,7</sup>, Jak<sup>8</sup>, phosphatidylinositol 3-kinase (PIK3)<sup>9</sup>, and tyrosine-protein kinase Src<sup>10</sup>. Inhibitors include the suppressor of cytokine signaling (Socs) family<sup>3,11,12</sup>, T cell protein tyrosine phosphatase (TC-PTP)<sup>13,14</sup>, receptor protein tyrosine phosphatase (RPTP)<sup>15</sup>, PTPN11/SHP2<sup>16</sup>, PTP-Meg2<sup>17</sup>, the protein inhibitor of activated Stat (PIAS) family<sup>18,19</sup>, and SIPAR (Stat3-interacting protein as a repressor)<sup>20</sup>. Protein tyrosine phosphatases (PTPs) are crucial negative regulators of Stat<sup>16,17</sup>. PTPs regulate Stat activity negatively either through dephosphorylation of protein tyrosine kinase Jak in the cytoplasm, e.g. PTPTC48<sup>21</sup>, or through direct dephosphorylation of Stat in the nucleus, e.g. by TC45<sup>14</sup>. Each PTP is capable of dephosphorylating multiple protein substrates. For example, TC45 mediates the dephosphorylation of Stat family members in the nucleus, such as Stat1, Stat3 and Stat5<sup>14,22,23</sup>.

In *Drosophila*, homologs of the mammalian Jak/Stat pathway components have been isolated, including ligands (Upds) encoded by the *unpaired (upd/os)* genes<sup>24</sup>, Domeless (Dome; also known as Mom)<sup>25</sup>, a Jak encoded by the *Hopscotch (hop)* gene<sup>26</sup> and a Stat (also known as *Stat92E*), encoded by *marelle (mrl)*<sup>27,28</sup>. Several negative regulators of Jak/Stat pathway are also found, such as suppressor of cytokine signaling at 36E (Socs36E)<sup>29</sup>, ken and barbie (ken)<sup>30</sup>, protein inhibitors of activated stats (PIAS)<sup>31</sup>, and nucleosome remodeling factor (NURF)<sup>32</sup>. In invertebrates, if protein tyrosine phosphatases participate in regulating Jak/Stat pathway is still not clear.

$\beta$ -Arrestins ( $\beta$ arrs) are multifunctional scaffold proteins that are involved in regulating desensitization and endocytosis of diverse cell surface receptors, such as G protein-coupled receptors<sup>33,34</sup>. Recent studies indicated

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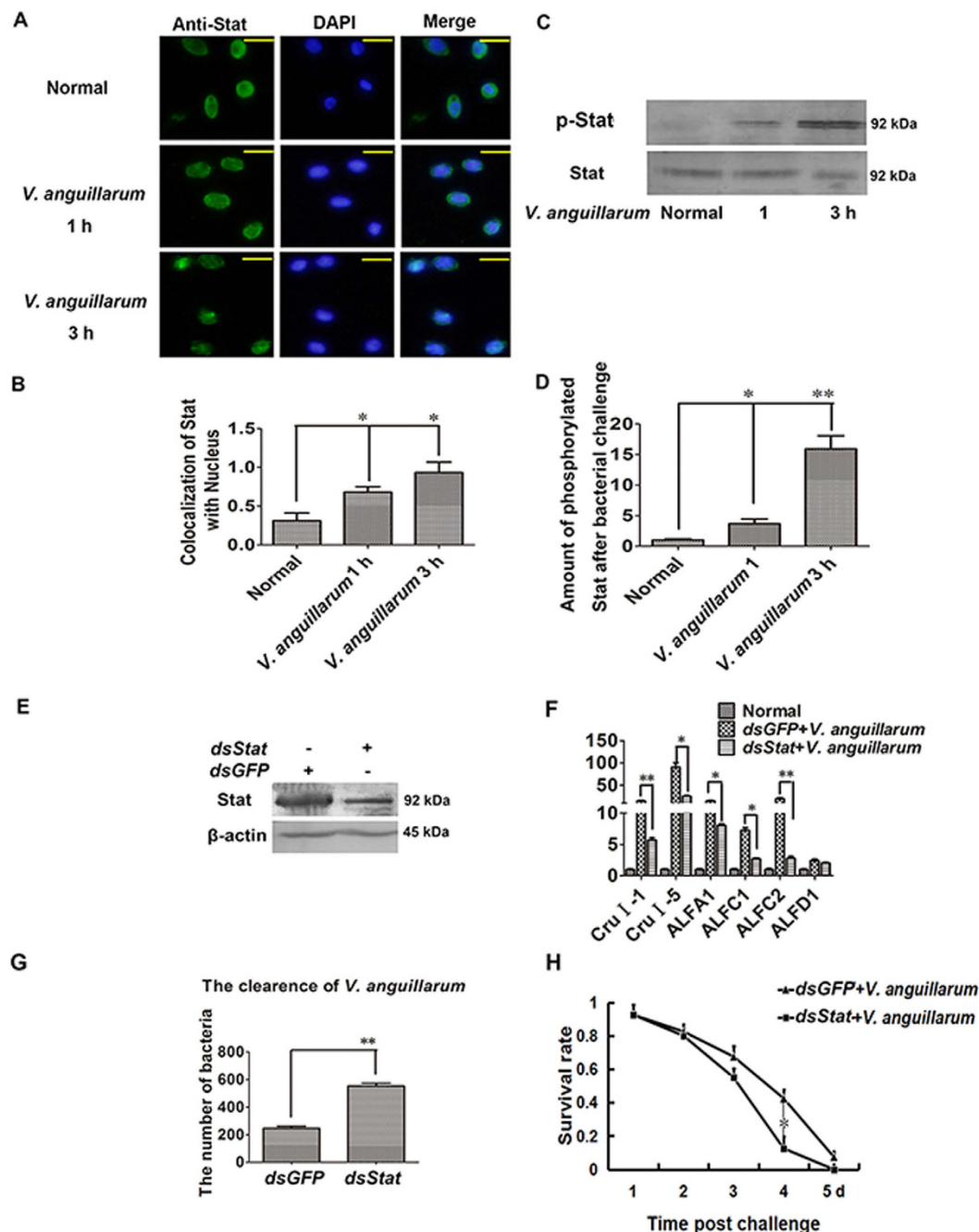
**Figure 1. Tissue distributions of  $\beta$ arr1,  $\beta$ arr2 and TC45 and Stat in shrimp.** (A–D) Tissue distributions of  $\beta$ arr1 (A),  $\beta$ arr2 (B), TC45 (C) and Stat (D) in shrimp, as analyzed by qRT-PCR (Upper panel) and western blotting (bottom panel).  $\beta$ -Actin was used as the control. The mRNA and proteins were extracted from hemocytes, heart, hepatopancreas, gills, stomach, and intestine and used for reverse transcription and western blotting. (E) Western blotting was used to indicate the molecular masses of native  $\beta$ arr1,  $\beta$ arr2, TC45, Stat and  $\beta$ -actin by detection using the corresponding polyclonal antibodies. The molecular weight markers were from Thermo Fisher Scientific, Lithuania. The experiments were repeats three times.

that nuclear  $\beta$ arr1 directly interacts with Stat1 in the nucleus after IFN- $\gamma$  treatment and accelerates Stat1 dephosphorylation by recruiting TC45 (the 45-kDa form of TC-PTP)<sup>35</sup>. The C-terminal region of the TC45 has a specific bipartite nuclear localization sequence that targets the enzyme to the nucleus<sup>36</sup>. Another study also found that GdX (X-linked gene in the G6PD cluster at Xq28, also known as Ubl4A, Ubiquitin-like protein 4A) promotes Stat3 dephosphorylation by mediating the interaction between TC45 and Stat3 specifically<sup>37</sup>. In shrimp, only one Stat similar to mammalian Stat5a/b<sup>38,39</sup> and two  $\beta$ arrs ( $\beta$ arr 1 and 2)<sup>40</sup> have been identified, but TC45 has not been reported. Furthermore, the functions of  $\beta$ arrs and TC45 in the Jak/Stat pathway remain unknown.

In this study, we showed that after bacterial infection, Stat phosphorylation increased, followed by its translocation into the nucleus to induce AMP expression in shrimp hemocytes.  $\beta$ Arr1, but not  $\beta$ arr 2, and TC45 accelerate the dephosphorylation of Stat in shrimp challenged by *V. anguillarum*. Further study showed that  $\beta$ arr1 recruits TC45 to phosphorylated Stat in the nucleus, and then mediates Stat dephosphorylation, leading to significantly decreased AMP expression in shrimp. Collectively, these results demonstrated that  $\beta$ arr1 acts as a scaffold protein to bridge the association between TC45 and Stat, which accelerates Stat dephosphorylation in the nucleus to moderate AMP expression in shrimp infected by *V. anguillarum*.

## Results

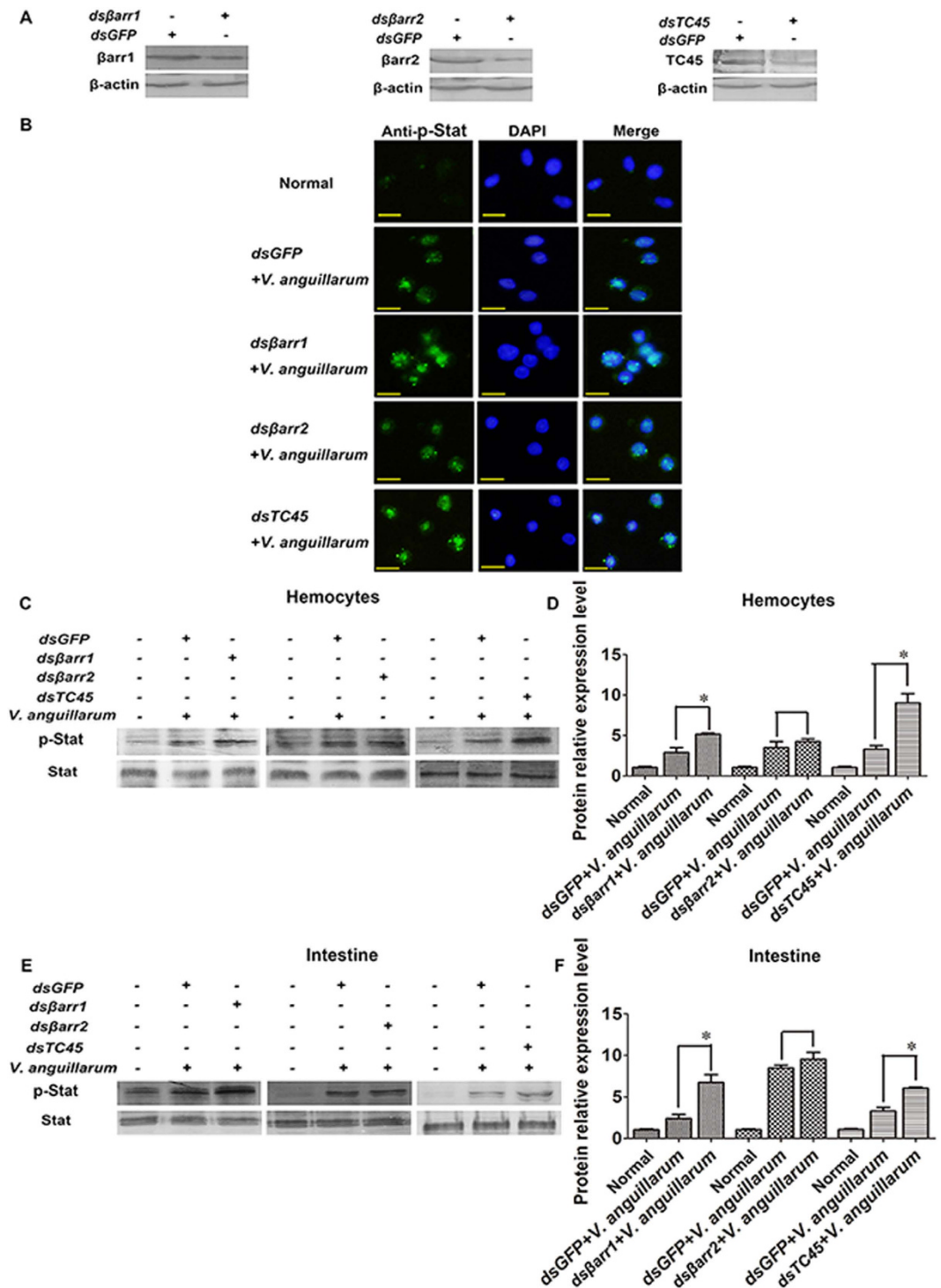
**Distributions of  $\beta$ arrs, TC45 and Stat in shrimp.**  $\beta$ arrs and Stat were reported in shrimp previously<sup>40,41</sup>. TC45 was first identified in shrimp and submitted to GenBank (no. KX358404). TC45 (51.06 kDa) contains a catalytic domain (22–292aa) and a nuclear localization signal at its non-catalytic C-terminal domain (293–451aa) with a sequence R  $\times$  RKR, which is similar to TC45 in mammals<sup>36</sup>. QRT-PCR and western blotting were used to analyze the distributions of  $\beta$ arr1,  $\beta$ arr2, TC45 and Stat transcripts and proteins. The results revealed that  $\beta$ arr1 was expressed in hemocytes, heart, hepatopancreas, gills, stomach and intestine (Fig. 1A).  $\beta$  Arr2 was mainly expressed in the hepatopancreas and stomach (Fig. 1B). TC45 was expressed in hemocytes and other all tested organs (Fig. 1C). Stat was expressed in all tested organs, but was expressed at a relatively low level in hemocytes (Fig. 1D). Molecular masses of native  $\beta$ arr1,  $\beta$ arr2, TC45, Stat and  $\beta$ -actin were confirmed by western blotting using their corresponding antibodies (Fig. 1E). These results suggested that  $\beta$ arr1,  $\beta$ arr2, TC45 and Stat are distributed ubiquitously in shrimp.



**Figure 2. Stat translocates into the nucleus to induce AMP expression against bacterial infection.**

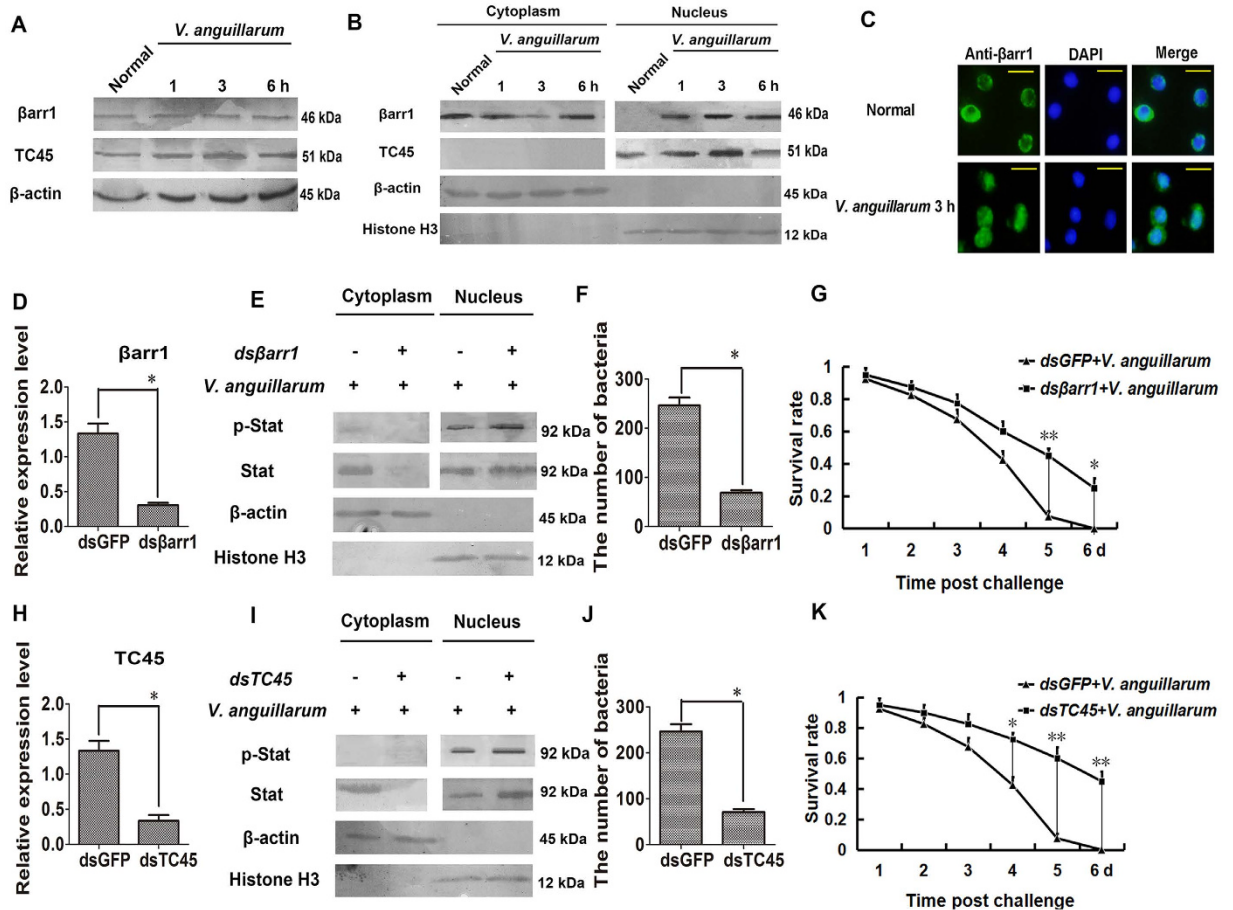
(A,B) Stat translocation in shrimp hemocytes challenged by *V. anguillarum* at 1 and 3 h was detected by immunocytochemistry using an anti-Stat antibody. (B) is the statistical analysis for the colocalization of Stat signal within the nucleus, as analyzed by WCIF ImageJ. (C,D) Stat phosphorylation in shrimp hemocytes challenged by *V. anguillarum* at 1 and 3 h, as detected by western blotting with an antibody recognizing phosphorylated Stat. (D) is the statistical analysis of C after digitization using Quantity One. (E) Western blotting was used to detect the RNAi efficiency of *dsStat* injection. (F) qRT-PCR detection of the expressions of *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1*, *ALF-C2* and *ALF-D1* in *Stat*-silenced shrimp challenged by *V. anguillarum* at 6 h. (G,H) The bacteria clearance and survival rate of *Stat*-silenced shrimp were analyzed. *dsGFP* injection was used as the control. The experiments were repeats three times. The significant differences between two groups were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests (\* $p < 0.05$ , \*\* $p < 0.01$ ).

**Stat is involved in antibacterial immunity.** To study the function of Stat, we firstly detected Stat translocation in hemocytes using immunocytochemistry, and found that Stat could translocate into the nucleus in shrimp hemocytes when challenged with *V. anguillarum* (Fig. 2A,B). The phosphorylation of Stat was detected



**Figure 3.**  $\beta$ Arr1 and TC45 reduce the phosphorylation of Stat. (A) The RNAi efficiency after *dsβarr1*, *dsβarr2* and *dsTC45* injection, as analyzed by western blotting. (B) Stat translocation in hemocytes was detected by immunocytochemistry using an antibody recognizing phosphorylated Stat in *βarr1*-, *βarr2*- or *TC45*-silenced shrimp challenged by *V. anguillarum*. (C–F) Stat phosphorylation in hemocytes (C,D) and the intestine (E,F) was detected by western blotting with the antibody recognizing phosphorylated Stat in *βarr1*-, *βarr2*- or *TC45*-silenced shrimp challenged by *V. anguillarum*. (D,F) separately, are the statistical analysis of (C,E) after digitization using Quantity One. *dsGFP* injection was used as the control. The experiments were repeats three times.

using anti-p-Stat (Abcam USA), which showed that phosphorylation of Stat in hemocytes was induced after challenge with *V. anguillarum* (Fig. 2C,D). The expression of AMPs regulated by Stat was also detected in *Stat* silenced shrimp, which showed that the expression of *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1* and *ALF-C2* was not

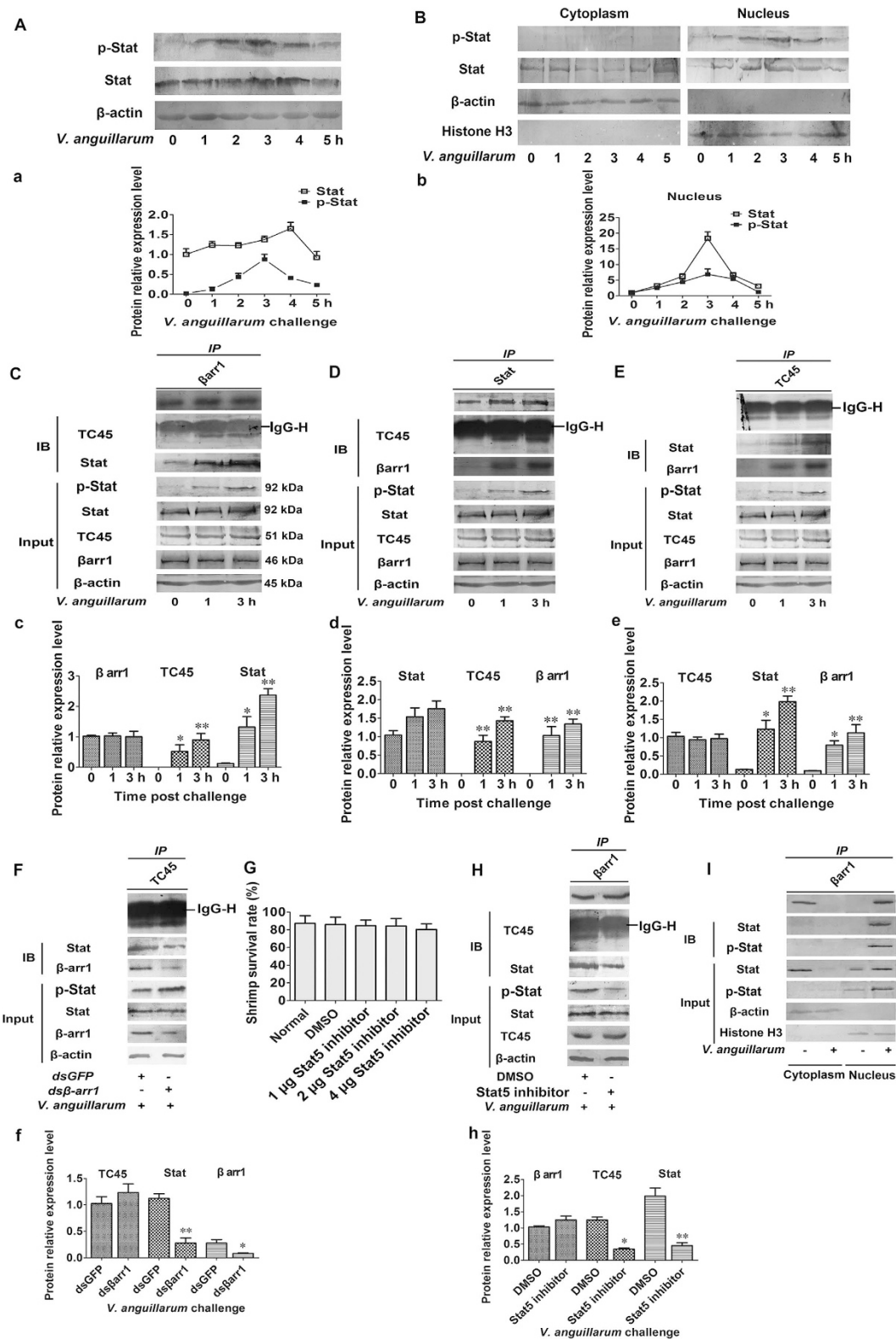


**Figure 4.  $\beta$ Arr1 and TC45 regulate negatively Stat's antibacterial function.** (A) The expressions of  $\beta$ arr1 and TC45 in the hemocytes of shrimp challenged with *V. anguillarum*, as analyzed by western blotting. (B) The distribution of  $\beta$ arr1 and TC45 in the nucleus and cytoplasm of hemocytes challenged by *V. anguillarum* at 1, 3, 6 h, as analyzed by western blotting. (C)  $\beta$ Arr1 translocated into nucleus of hemocytes from shrimp challenged by *V. anguillarum* at 3 h detected by immunocytochemistry using an  $\beta$ arr1 antibody. (D,H) The RNAi efficiency after *dsβarr1* and *dsTC45* injection, as analyzed by qRT-PCR. (E,I) Phosphorylated Stat in the hemocyte nucleus and cytoplasm, as detected by western blotting with an anti-Stat antibody in  $\beta$ arr1- or TC45-silenced shrimp challenged by *V. anguillarum*. *dsGFP* was used as the control. (F,J) Bacterial clearance was detected in  $\beta$ arr1- or TC45-silenced shrimp. *dsGFP* was used as the control. (G,K) The survival rates of  $\beta$ arr1- or TC45-silenced shrimp challenged by *V. anguillarum*. *dsGFP* was used as the control. The experiments were repeats three times. Differences between groups were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests. Significant differences are shown (\* $p < 0.05$ , \*\* $p < 0.01$ ).

induced in *Stat*-silenced shrimp (Fig. 2E,F). To further confirm whether *Stat* is involved in antibacterial immunity, the bacterial clearance and survival rates of shrimp were analyzed in *Stat*-silenced shrimp (Fig. 2G,H). The results showed that the count of *V. anguillarum* increased significantly in *Stat*-silenced shrimp (Fig. 2G). The survival rate of shrimp declined significantly in *Stat*-silenced shrimp compared with *GFP*-silenced shrimp (Fig. 2H). These results suggested that *Stat* plays an important role in anti-*V. anguillarum* immunity in shrimp.

**Stat phosphorylation increases in  $\beta$ arr1- and TC45-silenced shrimp.** *Stat* phosphorylation was analyzed in  $\beta$ arr1-,  $\beta$ arr2- or TC45-silenced shrimp to study whether  $\beta$ arr1,  $\beta$ arr2 and TC45 affect *Stat* phosphorylation (Fig. 3A). Immunohistochemistry showed that the level of phosphorylated *Stat* (p-*Stat*) in the nucleus increased in hemocytes of  $\beta$ arr1- and TC45-silenced shrimp, while there was almost no effect in  $\beta$ arr2-silenced shrimp challenged with *V. anguillarum* (Fig. 3B). Western blotting analysis in hemocytes also showed the same results as the immunocytochemistry (Fig. 3C,D). To further confirm the results, we detected the level of p-*Stat* in the intestines of  $\beta$ arr1-,  $\beta$ arr2- or TC45-silenced shrimp using western blotting. Same results were obtained as those in hemocytes (Fig. 3E,F). These results suggest that  $\beta$ arr1 and TC45, but not  $\beta$ arr2, inhibit *Stat* phosphorylation in shrimp.

**$\beta$ Arr1 and TC45 negatively regulate *Stat*'s antibacterial function by dephosphorylation of *Stat*.** To detect if  $\beta$ arr1 and TC45 are involved in *Stat*'s anti-*V. anguillarum* response, the protein expression



**Figure 5.**  $\beta$ Arr1 interacts with Stat and TC45 to form a heterotrimeric complex. (A-a) The total Stat and p-Stat protein level were detected in hemocytes after *V. anguillarum* challenge from 1 to 5 h. (a) is the statistical analysis of A after digitization using Quantity One. (B-b) Cytoplasmic and nuclear proteins extracted from hemocytes were used to detect the distribution of Stat and p-Stat in the nucleus and cytoplasm after *V. anguillarum* challenge from 1 to 5 h. (b) is the statistical analysis of B after digitization using Quantity One. (C-c) Co-IP was performed using an anti- $\beta$ arr1 antibody in hemocytes; TC45, Stat, and phosphorylated-Stat (p-Stat) were detected by their corresponding antibodies. (c) is the statistical analysis of C after digitization using Quantity One. (D-d) Co-IP was performed using an anti-Stat antibody in hemocytes; TC45,  $\beta$ arr1, and p-Stat were detected by their corresponding antibodies. (d) is the statistical analysis of D after digitization using

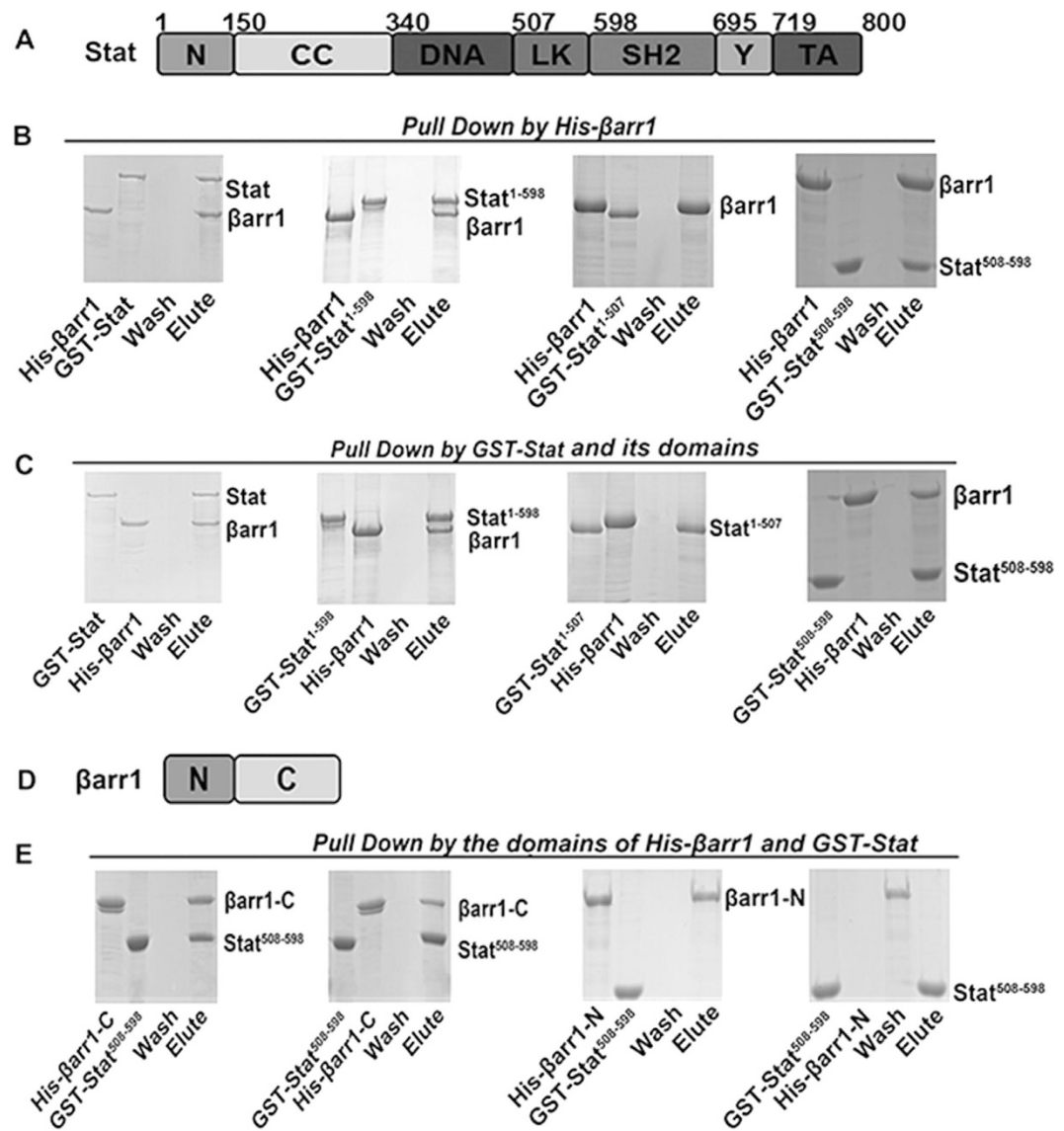
Quantity One. (E-e) Co-IP with an anti-TC45 antibody in hemocytes;  $\beta$ arr1, Stat and p-Stat were detected by their corresponding antibodies. (e) is the statistical analysis of E after digitization using Quantity One. (F-f) Co-IP with an anti-TC45 antibody using hemocytes from *βarr1*-silenced shrimp;  $\beta$ arr1, Stat, and p-Stat were detected by their corresponding antibodies. (f) is the statistical analysis of (F) after digitization using Quantity One. (G) The effect of Stat5 inhibitor on the viability of shrimp. Shrimp were treated with increasing concentrations of Stat5 inhibitor for 2 days and the survival rate was calculated. DMSO was used as the control. (H-h) Co-IP with an anti- $\beta$ arr1 antibody using hemocytes from the Stat5 inhibitor-injected shrimp; TC45, Stat, and p-Stat were detected by their corresponding antibodies. (h) is the statistical analysis of (H) after digitization using Quantity One. (I) Co-IP with an anti- $\beta$ arr1 antibody using cytoplasmic and nuclear proteins from hemocytes challenged with *V. anguillarum* at 3 h; Stat and p-Stat were detected by their corresponding antibodies. The experiments were repeats three times. Differences between groups were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests. Significant differences are shown (\* $p < 0.05$ , \*\* $p < 0.01$ ).

patterns, and subcellular distributions of  $\beta$ arr1 and TC45 were detected by western blotting after challenge with *V. anguillarum* at 1, 3 and 6 h. The results showed no change in the  $\beta$ arr1 expression pattern (Fig. 4A); however, it mainly existed in the cytoplasm in normal shrimp but increased in the nucleus in *V. anguillarum* challenged shrimp at 1, 3 and 6 h (Fig. 4B,C). The expression of TC45 increased at 3 h in *V. anguillarum* challenged shrimp and was present mainly in the nucleus (Fig. 4A,B). We then analyzed the level of p-Stat in hemocytes of *βarr1*-silenced and *TC45*-silenced shrimp (Fig. 4D,H). The results revealed that p-Stat in nucleus increased in *βarr1*- or *TC45*-silenced shrimp challenged by *V. anguillarum* (Fig. 4E,I). Bacterial clearance increased and the number of *V. anguillarum* decreased significantly in *βarr1*- or *TC45*-silenced shrimp (Fig. 4F,J). The survival rates of shrimp increased significantly in *βarr1*- or *TC45*-silenced shrimp compared with *GFP*-silenced shrimp (Fig. 4G,K). These results suggested that  $\beta$ arr1 and TC45 negatively regulate Stat's antibacterial function by dephosphorylating Stat.

**$\beta$ Arr1, TC45 and Stat interact with each other.** The expression pattern of total Stat and p-Stat in hemocytes after *V. anguillarum* challenge from 1 to 5 h was firstly analyzed by western blot. The results showed that total Stat was high expressed in unchallenged shrimp, but relatively increased from 3 to 4 h after bacterial challenge, and almost no p-Stat was detected in unchallenged shrimp, but the p-Stat was increased from 1 to 3 h and then recovered at 5 h after bacterial challenge (Fig. 5A-a). Then cytoplasmic and nuclear proteins from hemocytes were extracted respectively for western blot analysis, and the results showed that total Stat was existed in normal shrimp and decreased from 1 to 4 h and recovered at 5 h after bacterial challenge in cytoplasm. However, nearly no p-Stat was detected in cytoplasm of the hemocytes from normal and bacterial challenged shrimp (Fig. 5B). Conversely, the expression of total Stat was increased from 1 to 4 h after *V. anguillarum* challenge in nucleus, and p-Stat was also increased from 1 to 3 h and then decreased from 4 to 5 h post bacterial challenge in nucleus (Fig. 5B-b). These results suggested that bacterial challenge induced Stat phosphorylation and translocation into nucleus leading to a clear increase of p-Stat in nucleus at early stage of bacterial challenge, and the p-Stat was dephosphorylated leading to obvious decrease at late stage of bacterial challenge. To analyze possible mechanism, the Co-IP assays were performed to study the interactions of  $\beta$ arr1 and TC45 with Stat in hemocytes using antibodies against  $\beta$ arr1, TC45 or Stat. The results showed that  $\beta$ arr1, TC45 and Stat interacted with each other and the interaction was enhanced following the increase of Stat phosphorylation (Fig. 5C-E). The interaction of TC45 with Stat became weak in *βarr1*-silenced shrimp after challenge with *V. anguillarum* (Fig. 5F-f). To further confirm above results, Stat5 inhibitor was used to inhibit Stat phosphorylation. Initially, the toxic effect of Stat5 inhibitor on shrimp was determined by analyzing the survival rate of shrimp after Stat5 inhibitor injection. The results showed that low doses of Stat5 inhibitor ( $< 4 \mu\text{g}$  per shrimp) did not reduce the viability of shrimp (Fig. 5G). After Stat5 inhibitor injection, Co-IP with  $\beta$ arr1 antibody was performed using hemocytes. The results showed that the interaction of  $\beta$ arr1, TC45 and Stat became weak after injection of Stat5 inhibitor (Fig. 5H-h). As p-Stat could be translocated into the nucleus, the nuclear and cytoplasmic proteins were extracted and the Co-IP assays were performed. The result showed  $\beta$ arr1 could interact with Stat in the nucleus but not in the cytoplasm (Fig. 5I). The results further confirmed that the nuclear  $\beta$ arr1 directly interacts with Stat. The above results suggested that  $\beta$ arr1 acts as a scaffold protein to recruit the tyrosine phosphatase TC45 onto phosphorylated Stat to dephosphorylate Stat in the nucleus.

**The C-terminal domain of  $\beta$ arr1 interacts with the Link domain of Stat.** *M. japonicus* Stat contains N-terminal domain (NTD; aa 1–150), a coiled-coil domain (CC; aa 151–340), a DNA-binding domain (DB; aa 341–507), a linker domain (LK; aa 508–598), an SH2 domain (SH2; aa 599–695), a tyrosine phosphorylation site (Y site; aa 696–719) and a transactivation domain, TAD, (aa 720–800) (Fig. 6A). To detect which domain of Stat is responsible for the interaction with  $\beta$ arr1, recombinant proteins of Stat and its individual domains with GST-tags (Stat, 1–598, 1–507, 508–598) (Fig. 6A), and  $\beta$ arr1 and its different domains with His-tags  $\beta$ arr1,  $\beta$ arr1-N (N-terminal domain),  $\beta$ arr1-C (C-terminal domain) (Fig. 6D) were used in pull-down assays. As shown in Fig. 6B,C, the LK domain of Stat interacted with  $\beta$ arr1. Recombinant  $\beta$ arr1,  $\beta$ arr1-N and  $\beta$ arr1-C were used for pull-down assays to analyze the interaction with LK domain of Stat (Fig. 6E). The results showed that LK domain of Stat interacts with the C-terminal domain of  $\beta$ arr1.

**TC45 interacts with Stat<sup>599–800</sup> and the C-terminal domain of  $\beta$ arr1.** To study which domain of Stat interacts with TC45, His-tagged TC45 was also expressed in *E. coli*. The results showed TC45 could interact with Stat<sup>599–800</sup> which contains the Tyr phosphorylation sites (Fig. 7B). We expressed TC45 with a GST-tag, and



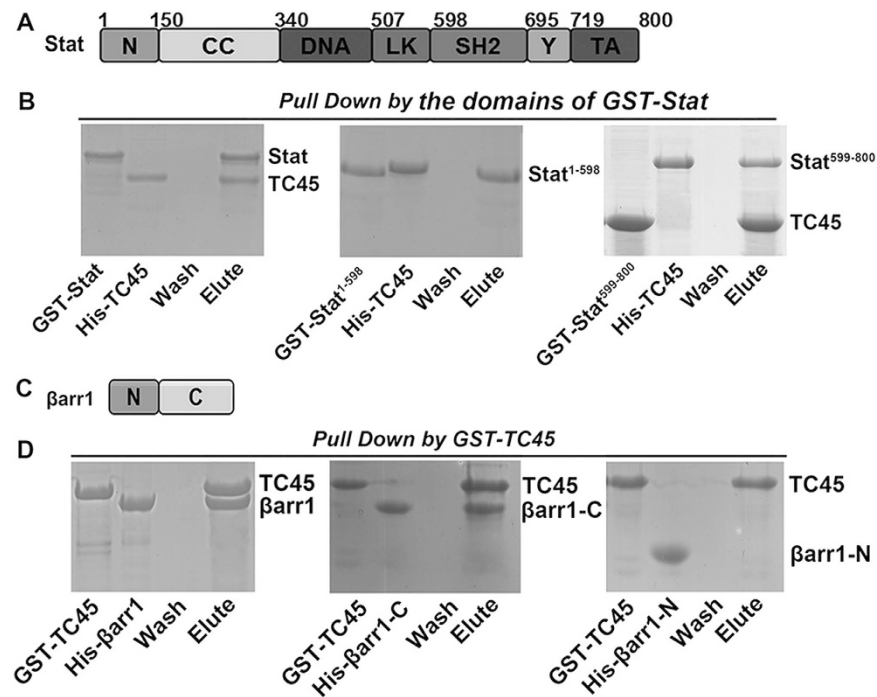
**Figure 6.**  $\beta$ Arr1 interacts with Stat. (A) Domain architectures of Stat. (B) His-pull-down assays were performed to detect the interaction of His- $\beta$ arr1 with different domains of GST-Stat. (C) GST-pull-down assays were performed to detect the interaction of different domains of GST-Stat with His- $\beta$ arr1. (D) Domain architectures of  $\beta$ arr1. (E) His- and GST-pull-down assays were performed to detect interaction of His- $\beta$ arr1-C with Stat<sup>508-598</sup> of GST-Stat. The experiments were repeats three times.

GST-pull-down with  $\beta$ arr1 was performed. The results showed  $\beta$ arr1 interacted with TC45 via its C-terminal domain (Fig. 7D). Therefore,  $\beta$ arr1 could interact with the LK domain of Stat and with TC45 via its C-terminal domain.

**$\beta$ Arr1 and TC45 affect the expressions of AMPs that are regulated by Stat.** To study if the AMPs regulated by Stat are also affected by  $\beta$ arr1 and TC45, the expression of five AMPs was detected in  $\beta$ arr1- or TC45-silenced shrimp challenged with *V. anguillarum* (Fig. 8A,B). The results showed that the expression of *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1*, and *ALF-C2* increased significantly in  $\beta$ arr1- or TC45-silenced shrimp after challenge with *V. anguillarum* at 6 h (Fig. 8C,D).

To further confirm the results,  $\beta$ arr1- or TC45-overexpression assays were conducted. The results showed that Stat phosphorylation was inhibited in  $\beta$ arr1- or TC45-overexpressing shrimp challenged with *V. anguillarum* (Fig. 8E,F). The expression of *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1*, and *ALF-C2* declined significantly in  $\beta$ arr1- and TC45-overexpressing shrimp after challenge with *V. anguillarum* at 6 h (Fig. 8G,H). These results suggested that  $\beta$ arr1 and TC45 negatively regulate AMP expression.





**Figure 7.** TC45 interacts with Stat and  $\beta$ arr1. (A) Domain architecture of Stat. (B) GST-pull-down assays were performed to detect the interaction of GST-TC45 with different domains of His-Stat. (C) Domain architecture of  $\beta$ arr1. (D) The interaction of GST-TC45 with different domains of His- $\beta$ arr1 was analyzed by a pull-down assay. The experiments were repeats three times.

## Discussion

Stats, as important transcription factors, are regulated tightly in animals. In *Drosophila*, Stat92E is activated by Upds, Dome, Jak and inhibited by Socs36E<sup>29</sup>, ken<sup>30</sup>, PIAS<sup>31</sup>, and NURF<sup>32</sup>. SOCSs regulate Stat activation by inhibiting Jak<sup>29</sup>. Ken and NURF inhibit Stat activation in the nucleus by directly blocking the binding of Stat to its targets<sup>30</sup>. PIAS can interact directly with p-Stat to inhibit Stat activation<sup>31</sup>. In shrimp, a Dome<sup>42</sup>, a Jak<sup>43</sup> and a Stat<sup>38,39</sup> in the Jak/Stat pathway have been identified. Shrimp Stat is similar to mammalian Stat5a/b. In mammals, Jak2 and 3<sup>44,45</sup> regulate Stat5a/b and the inhibitors are Socs2<sup>46</sup>, TC-PTP<sup>47</sup> and PTP1B<sup>48</sup>. The inhibitor SOCS2 has also been cloned in shrimp<sup>38</sup>. In the present study, we found that Stat was activated by *V. anguillarum* challenge and the activated Stat was negatively regulated by  $\beta$ arr1 and TC45 in shrimp.

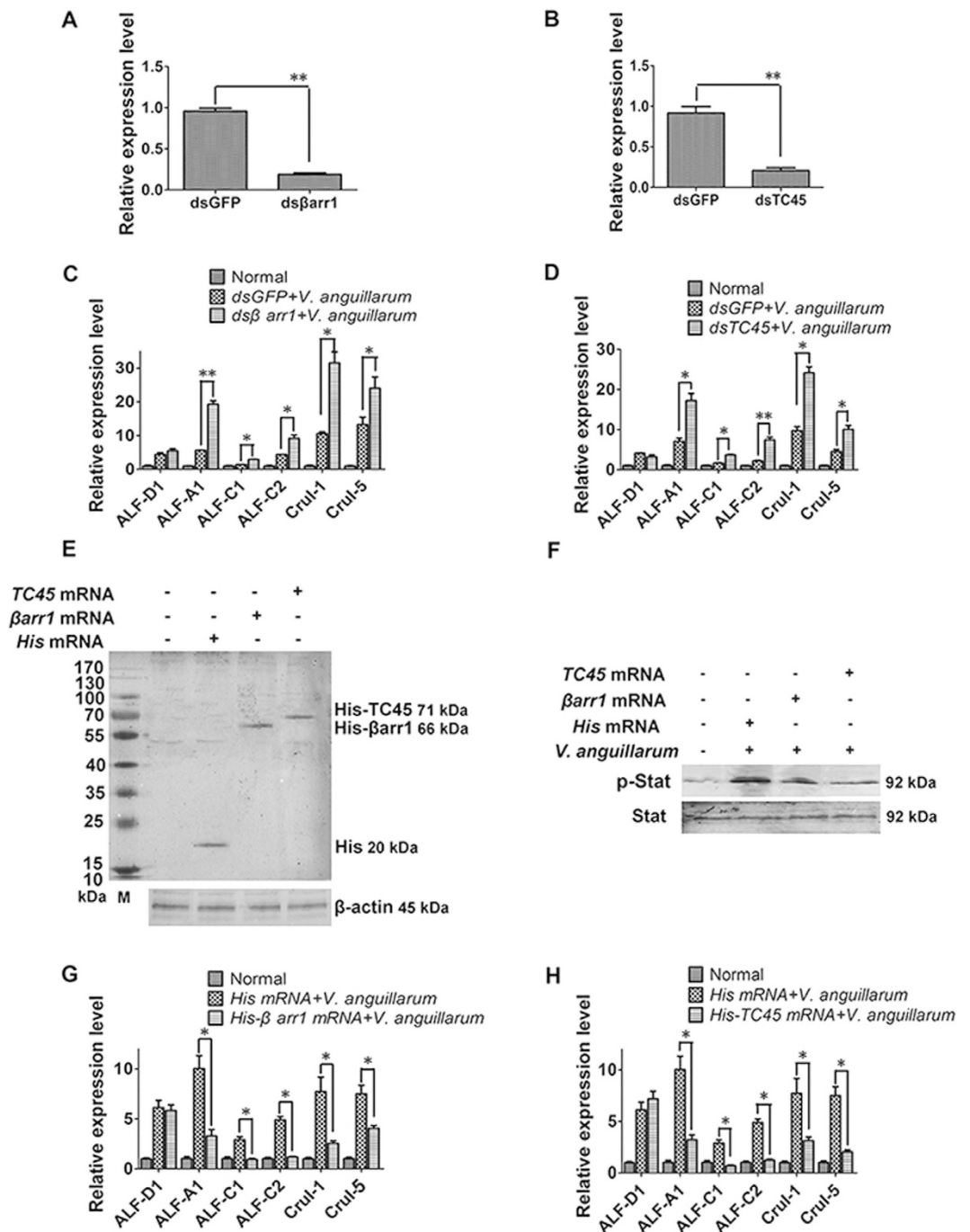
$\beta$ Arrests are multifunctional signaling molecules that can affect multiple signal pathways, such as GPCR signal transduction<sup>33</sup>, and the activities or subcellular distribution of signaling molecules<sup>34</sup>. Recent studies suggested that  $\beta$ arr1 in the nucleus binds and recruits histone acetylase p300 to specific genome promoters and regulates gene transcription in mammals<sup>49</sup>. Moreover, in mammals,  $\beta$ arr1 also recruits tyrosine phosphatase TC45 to Stat1 in the nucleus in response to IFN- $\gamma$  stimulation and affects Stat1 phosphorylation<sup>35</sup>. In shrimp, two  $\beta$ -arrestins ( $\beta$ arr 1 and 2) were identified that regulated the Toll pathway negatively by inhibiting Dorsal translocating into the nucleus<sup>40</sup>. In the present study,  $\beta$ arr1, but not  $\beta$ arr2, was observed to regulate specifically tyrosine phosphorylation of Stat in shrimp, resulting in decreased AMP expression.  $\beta$ Arr1 is a scaffold protein; therefore, how does it function to regulate tyrosine phosphorylation of Stat?

TC45 is the most important tyrosine phosphatase in the process of Stat1 dephosphorylation in mammals<sup>23</sup>. PTPs regulate Stat3 activity negatively, either in the cytoplasm, through dephosphorylating protein tyrosine kinase Jak (for instance, PTPC48)<sup>21</sup>, or in the nucleus, through directly dephosphorylating Stat3 (for instance, TC45)<sup>14</sup>. Moreover, TC45 is not a Stat3-specific phosphatase; it also mediates the dephosphorylation of other Stat family members such as Stat1<sup>22,23</sup>. GdX, as a TC-PTP cofactor, bridges the association of TC45 with Stat3 specifically to mediate Stat3 dephosphorylation, which blocks Stat3-P-dependent cancer cell growth in mammals<sup>37</sup>. However, whether tyrosine phosphatase TC45 could affect Stat dephosphorylation in invertebrate nucleus is still unknown. In this paper, we found that in the nucleus, TC45 could interact with Stat in nucleus to dephosphorylate Stat, resulting in decreased AMP expression.

In summary, *V. anguillarum* infection activates Stat signaling by inducing tyrosine phosphorylation of Stat. p-Stat is then translocated into the nucleus to regulate AMP expression. The scaffold protein  $\beta$ arr1 bridges TC45 and Stat in nucleus, allowing TC45 to dephosphorylate Stat, thereby inhibiting Stat's transcription activity (Fig. 9). Our results demonstrated that in shrimp, TC45 and  $\beta$ arr1 are negative regulators in Stat signaling.

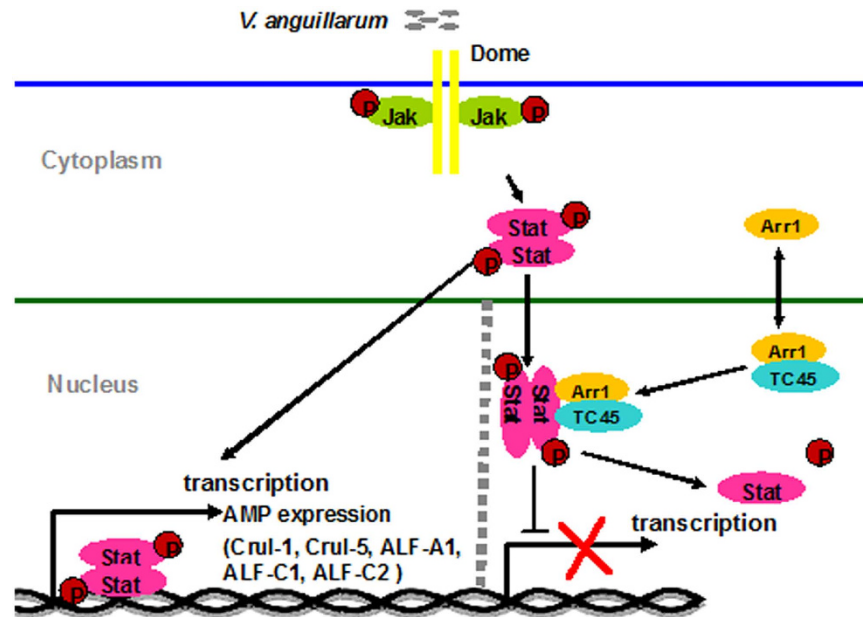
## Materials and Methods

**Tissue distribution.** Kuruma shrimp *Marsupenaes japonicus* (8–10 g each) from a fishery market in Jinan, Shandong Province, China were cultured in laboratory aquarium tanks with aerated seawater at 22 °C. After



**Figure 8.**  $\beta$ Arr1 and TC45 are involved in the regulation of AMP expression. (A,B) RNAi efficiency after *dsβarr1* and *dsTC45* injection, as detected by qRT-PCR. (C,D) qRT-PCR to detect the expression of *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1*, *ALF-C2* and *ALF-D1* in  $\beta$ arr1 or TC45-silenced shrimp challenged by *V. anguillarum* at 6 h. (E) Western blotting to analysis of  $\beta$ arr1 or TC45 expression in hemocytes from mRNA-injected shrimp. Hemocytes were extracted for western blotting 24 h post mRNA injection. An anti-His-tag monoclonal antibody was used as the primary antibody. (F) Stat phosphorylation was detected in  $\beta$ arr1- and TC45-overexpressing shrimp after challenge with *V. anguillarum* at 3 h. (G,H) qRT-PCR was used to detect the expressions of *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1*, *ALF-C2* and *ALF-D1* in  $\beta$ arr1- and TC45-overexpressing shrimp challenged by *V. anguillarum* at 6 h. The experiments were repeats three times. Differences were analyzed using one-way ANOVA, followed by Tukey's multiple comparison tests. The significant differences are shown (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).

acclimatization for 2 days, five organs from at least three normal shrimp, including the heart, hepatopancreas, gills, stomach and intestine, were collected. Total RNA was extracted using the Trizol reagent (Cwbio, Beijing,



**Figure 9.** A model of Stat signaling attenuation in the nucleus. Stat phosphorylation and transcription are activated by *V. anguillarum*, followed by induction of AMP (Cru1-1, Cru1-5, ALF-A1, ALF-C1, ALF-C2) expression.  $\beta$ Arr1 interacts with TC45 to dephosphorylate Stat in the nucleus. Thus, the TC45/ $\beta$ arr1 complex promotes Stat dephosphorylation to inhibit Stat transcription activity, thereby attenuating AMP expression in the nucleus.

China) and proteins were obtained using a lysis buffer (150 mM NaCl, 1.0% Nonident-P40, 0.1% SDS, 50 mM Tris [pH 8.0] containing a protease inhibitor cocktail (Abcam USA). For hemocytes collection, the hemolymph was extracted from at least three shrimp using a syringe containing 1 ml of anticoagulant buffer (0.45 M NaCl, 10 mM KCl, 10 mM EDTA and 10 mM HEPES, pH 7.45) and immediately centrifuged at  $800 \times g$  for 10 min ( $4^\circ\text{C}$ ).

The mRNA distribution of four genes,  $\beta$ arr1,  $\beta$ arr2, TC45 and Stat, in hemocytes, heart, hepatopancreas, gills, stomach and intestine was analyzed using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) with the primers RT-F and RT-R for the above four genes (Table 1).  $\beta$ -actin was used as the control with the primers  $\beta$ -actinF and  $\beta$ -actinR (Table 1). All experiments were repeated at least three times using individual templates. The qRT-PCR program was:  $95^\circ\text{C}$  for 5 min; followed by 40 cycles at  $95^\circ\text{C}$  for 10 s,  $60^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 20 s. The plate was read at  $78^\circ\text{C}$  for each cycle. The final product was analyzed via a DNA melting analysis from  $65^\circ\text{C}$  to  $95^\circ\text{C}$ . The obtained data were analyzed statistically and their relative expressions were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method, as described previously<sup>50</sup>. Differences in the unpaired sample *t* test were considered significant at  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ . The proteins of  $\beta$ arr1,  $\beta$ arr2, TC45 and Stat from hemocytes and five organs were analyzed by western blotting with their corresponding antibodies.

**Molecular cloning and sequence analysis.** The full-length cDNA sequences of TC45 and Stat were obtained from transcriptomic sequencing of *M. japonicus*. The sequences of  $\beta$ arr1 and  $\beta$ arr2 were reported in our previous paper<sup>40</sup>. Similarity analysis was conducted using BLASTx (<http://www.ncbi.nlm.nih.gov/>). The cDNA sequences TC45 and Stat were conceptually translated and the corresponding deduced proteins were predicted using ExpPASy (<http://www.expasy.org/>). The domain architecture prediction of the proteins was analyzed using SMART (<http://smart.embl-heidelberg.de/>).

**Recombinant expression and antiserum preparation.** *M. japonicus* Stat contains an NTD, CC, DB, LK, SH2, Y site and TAD. DNAs encoding the full length Stat, and fragments 1–507, 1–598, 508–598 and 599–800 of Stat were recombinantly and separately expressed in *Escherichia coli* as GST-tagged proteins.  $\beta$ arr1,  $\beta$ arr1-N,  $\beta$ arr1-C and  $\beta$ arr2 were recombinantly expressed with His-tags and GST-tags; TC45 was recombinantly expressed as a GST-tagged protein, all in *E. coli*. The sequences encoding the above proteins were amplified using the primers ExF and ExR (Table 1). The PCR products were inserted into vector pET32a (Novagen) or pGEX4T-1 (GE Healthcare). The recombinant proteins were purified by affinity chromatography using His-Bind resin ( $\text{Ni}^{2+}$ -resin; Novagen, Darmstadt, Germany) or GST-resin (GenScript, Nanjing, China), following the manufacturers' instructions. Antiserum preparation was performed as previously described<sup>51</sup>.

**RNA interference.** The cDNA fragments of  $\beta$ arr1,  $\beta$ arr2, TC45 and Stat amplified by primers Fi and Ri (Table 1) were used as templates for the synthesis of dsRNA (double strand RNA). The cDNA fragment of GFP used for dsGFP synthesis was amplified using primers GFP-Fi and GFP-Ri (Table 1). The dsRNA was synthesized using T7 polymerase (Fermentas, USA) based on a previous method<sup>52</sup>. dsRNA ( $20\ \mu\text{g}$ ) of  $\beta$ arr1,  $\beta$ arr2, TC45 or Stat was injected into the abdominal segment of shrimp. To enhance the RNAi effect, a second injection was

Primer	Sequence (5'-3')
Tissue distribution and expression pattern analysis	
$\beta$ arr1-RT-F	TTTCACGCTGACGCCACT
$\beta$ arr1-RT-R	AGCAACCAGATCACCAACTAG
$\beta$ arr2-RT-F	TGGCTCTATTCCCTCTGCG
$\beta$ arr2-RT-R	TGGCTCTATTCCCTCTGCG
TC45-RT-F	CTCCCTTACCCGCTCTATGA
TC45-RT-R	TTTCTCTGCAAATGTTCTGT
Stat-RT-F	GGTCCCAGTTCTGTAAAGG
Stat-RT-R	TAGGCACATTCCGATAAA
$\beta$ -actin F	CAGCCTTCCTTCCTGGGTATGG
$\beta$ -actin R	GAGGGAGCGAGGGCAGTGATT
ALF-A1-RT-F	CTGGTCGGTTTCCTGGTGGC
ALF-A1-RT-R	CCAACCTGGGCACCACATACTG
ALF-C1-RT-F	CGCTTCAAGGGTCGGATGTG
ALF-C1-RT-R	CGAGCCTCTTCCTCCGTGATG
ALF-C2-RT-F	TCCTGGTGGTGGCAGTGGCT
ALF-C2-RT-R	TGCGGGTCTCGGCTTCTCCT
ALF-D1-RT-F	GCTTTTATTTTGGGGGTACGCTGT
ALF-D1-RT-R	CTTTGGCGTGGAACAAGGTAGAGGAT
Cru1-1-RT-F	TGCTCAGAACTCCCTCCACC
Cru1-1-RT-R	TTGAATCAGCCCATCGTCG
Cru1-5-RT-F	TGCGAAACAGACAGGGATTGC
Cru1-5-RT-R	CCGAAGACCAGATGACCGAAA
Recombinant expression	
$\beta$ arr1-ExF	TACTCAGGATCCATAATGGAGGACAACAG
$\beta$ arr1-ExR	TACTCACTCGAGTTCCTACGCCTCTGT
$\beta$ arr1-C-ExF	TACTCAGGATCCATAATGGAGGACAACAGC
$\beta$ arr1-C-ExR	TACTCACTCGAGGCATACATGACCTTCCTG
$\beta$ arr1-N-ExF	TACTCAGGATCCCAAGGAGAACAGCCAAGTGTG
$\beta$ arr1-N-ExR	TACTCACTCGAGTTCCTACGCCTCTGTTTCTCC
TC45-ExF	TACTCAGAATTCTGTCAGTATGCCTACAAATCTA
TC45-ExR	TACTCACTCGAGCTGGACACGAGGACGCTT
Stat(1aa)-ExF	TACTCAGAATTCTGTCGTTGTGGAACAGAGC
Stat(800aa)-ExR	TACTCACTCGAGTTACAAATGCCGGTGAACATA
Stat(507aa)-ExR	TACTCACTCGAGAGCATTATCCCATGA
Stat(507aa)-ExF	TACTCAGGATCCACAGTGTGTCATGGGAT
Stat(598aa)-ExR	TACTCACTCGAGCAGCATCTCTTCAGC
Stat(598aa)-ExF	TACTCACTCGAGAAGTACTACACGCCA
RNA interference	
$\beta$ arr1-Fi	GCGTAATACGACTCACTATAGGCTGATTCAAGTCAAAGGAG
$\beta$ arr1-Ri	GCGTAATACGACTCACTATAGGGAATCTTCTTTCTGTTCAAC
$\beta$ arr2-Fi	GCGTAATACGACTCACTATAGGTCCTCTGCGTGAACATTTTCC
$\beta$ arr2-Ri	GCGTAATACGACTCACTATAGGCTTCTCTATATCTCCTCGA
TC45-Fi	GCGTAATACGACTCACTATAGGATGGTGCACATAAGCTA
TC45-Ri	GCGTAATACGACTCACTATAGGCACAATTCATGTAGGA
Stat-Fi	GCGTAATACGACTCACTATAGGGACTTTCCTGCTCCGTTTC
Stat-Ri	GCGTAATACGACTCACTATAGGGCGTTGGCACTGTTGAGAC
GFP-Fi	GCGTAATACGACTCACTATAGGTGGTCCCAATTCTCGTGAAC
GFP-Ri	GCGTAATACGACTCACTATAGGCTTGAAGTTGACCTTGATGCC

**Table 1. Sequences of the primers used in this study.**

performed 12 h after the first injection. The *dsGFP* was used as the control. The hemocytes were collected from the shrimp 24 h after the second injection, and total RNA was extracted for RNAi efficiency analysis by RT-PCR using primers RT-F and RT-R (Table 1). Total protein was also extracted. The experiments were repeated three times.

**Bacterial clearance and Survival rate assay.** The bacterial clearance assay was performed in  $\beta$ arr1-, TC45- and Stat-silenced shrimp. Shrimp were separated into five groups, and injected with *ds* $\beta$ arr1, *ds*TC45 and *ds*Stat, or injected with *dsGFP* as a control. The four groups were then injected with *V. anguillarum* (20  $\mu$ l,

$1 \times 10^8$  CFU). One hour after bacteria injection, shrimp hemolymph was collected and diluted in PBS (140 mM NaCl, 10 mM sodium phosphate, pH 7.4) (1/10000), and then cultured on LB solid plates overnight in 37 °C. The number of bacterial colonies was counted. The assay was repeated three times.

For the survival assay, shrimp (8–10 g each individual, 30 shrimp per group) were divided into four groups, and injected with *dsβarr1*, *dsTC45* or *dsStat*; The *dsGFP* injection group was used as the control. The gene-silenced shrimp and the control group were infected with *V. anguillarum* (20 μl,  $1 \times 10^8$  CFU) and shrimp mortality was detected. The number of dead shrimp was monitored every day. The survival rate of the each group was calculated. The experiments were repeated three times.

**Western blotting analysis.** Proteins were extracted from the hemocytes and intestine of normal shrimp and bacterial challenged shrimp. Cytoplasmic and nuclear proteins were extracted, the latter using a Nuclear Protein Extraction Reagent Kit (BioTeke, China), following the manufacturer's instruction. The obtained samples were separated by 10% SDS-polyacrylamide gel electrophoresis. The proteins in the gel were then transferred onto a nitrocellulose membrane. The membrane was then blocked for 2 h with 3% non-fat milk in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% Tween-20), and incubated with 1/100 diluted antiserum against βarr1, βarr2, TC45, Stat, p-Stat or β-actin in TBST with 3% non-fat milk for 3 h. Alkaline phosphatase-conjugated goat anti-rabbit IgG (1/10,000 diluted in TBS) was added after washing out nonspecific binding antiserum with TBST and incubation continued for 2 h. The membrane was dipped in the reaction system (10 ml of TBS, with 45 μl of NBT and 35 μl of BCIP) in the dark for 5–20 min at 37 °C to visualize the signal.

**Immunocytochemistry.** Hemolymph from normal or challenged shrimp was fixed with a 1 ml mixture of anticoagulant (pH 7.4) and 4% paraformaldehyde (1:1) and then centrifuged at  $600 \times g$  for 3 min at 4 °C. Collected hemocytes were washed three times with PBS and then deposited on a glass slide, washed with PBS and then incubated in 0.2% triton at 37 °C for 5 min. After washing with PBS, the hemocytes on the glass slide were blocked with 3% BSA (30 min, 37 °C), and incubated with an antibody recognizing phosphorylated Stat (anti-p-Stat) (1:400 in 3% BSA) overnight at 4 °C. On the second day, the glass slide with the hemocytes was washed with PBS, and incubated with 3% BSA for 10 min, and then with Alexa fluor 488-conjugated anti-rabbit secondary antibody (1:800 ratio, diluted in 3% BSA) for 1 h at 37 °C in the dark. After washing six times, the hemocytes were incubated with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI, AnaSpec Inc., San Jose, CA, USA; 1 μg/ml in PBS) for 10 min at room temperature and then washed six times. Hemocytes on the slide were observed under an Olympus BX51 fluorescence microscope (Shinjuku-ku, Tokyo, Japan).

**Co-immunoprecipitation (Co-IP) assay.** Proteins from hemocytes were collected with lysis buffer containing a protease inhibitor cocktail (Merck, Gemery) and incubated with protein A for 10 min to remove non-specific binding proteins. The proteins were then incubated with the antibodies of interest overnight at 4 °C. On the second day, the mixture (protein and antibody) was incubated with protein A for 3 h at room temperature and then washed with PBS four times. The obtained mixture (bound protein, antibody and protein A) was analyzed by western blotting.

**Inhibitor injection.** Stat from shrimp is similar to human Stat5; therefore, Stat5 inhibitor from Merck was used to inhibit shrimp Stat. To ascertain the effect of Stat5 inhibitor on shrimp viability, the survival rate of shrimp after inhibitor injection was analyzed. Shrimp (8–10 g each individual, 20 shrimp per group) were divided into four groups, and injected with 1, 2 and 4 μg of Stat5 inhibitor, respectively. The dimethylsulfoxide (DMSO) injection group was used as the control. For the Co-IP assay, Stat5 inhibitor (2 μg) was injected into each shrimp, which were then challenged with *V. anguillarum*. DMSO injection was used as a control. The hemocyte proteins were collected for protein extraction 3 h after *V. anguillarum* challenge to further detect if Stat inactivation affected the interaction of βarr1 with Stat and TC45.

**Pull-down assays.** The recombinant proteins tagged with a GST-tag (30 μg) were added into 20 μl of Glutathione resin and incubated for 2 hours at room temperature with slight rotation. The mixture (resin and binding protein) was washed with TBS three times by centrifugation at  $500 \times g$  for 3 min to remove the unbound proteins. The His-tagged test protein was added into the mixture containing the Glutathione resin, and gently rotated at room temperature for 2 h. After washing three times, the mixture was analyzed by SDS-PAGE. The pull-down assay for His-tag proteins was performed the same as that for GST-tagged proteins.

**AMP expression in Stat-, βarr1- and TC45-silenced shrimp challenged with *V. anguillarum*.** Shrimp were divided into four groups and injected with *dsGFP*, *dsStat*, *dsβarr1* and *dsTC45*, separately. AMPs including *CruI-1*, *CruI-5*, *ALF-A1*, *ALF-C1*, *ALF-C2* and *ALF-D1*<sup>53</sup> were detected by qRT-PCR with corresponding primers (Table 1) after 6 h of *V. anguillarum* challenge. Shrimp injected with *dsGFP* were used as the control.

**Overexpression of βarr1 and TC45.** The pET32a vectors containing the *βarr1* and *TC45* genes were digested with restriction enzyme *XhoI*. The cleaved vectors were used as template to transcribe the capped *βarr1* and *TC45* mRNA using ta T7 RNA polymerase *in vitro* transcription kit (Ambion, Inc.) according to the manufacturer's instructions. The empty pET32a vectors were also digested by *XhoI* and used as a template to transcribe Trx-tag and His-tag sequences for use as controls. The mRNA for *βarr1* and *TC45* (at a concentration of 6 μM) with His-tag sequences and the control sequences were injected separately into shrimp. After 24 h of mRNA injection, the hemocyte proteins of each group were extracted for western blotting analysis using an anti-His-tag monoclonal antibody to detect the efficiency of overexpression. The proteins extracted from hemocytes were also used for western blotting to detect Stat phosphorylation in *βarr1*- or *TC45*-overexpressing shrimp after challenge

with *V. anguillarum* for 3 h. The *V. anguillarum* were injected into shrimp 6 h post mRNA injection, and total RNA was isolated from shrimp hemocytes using the Trizol reagent and used for reverse transcription of cDNA to detect AMP expression.

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## Author Contributions

Conceived and designed the experiments: J.-X.W., J.-J.S. and X.-F.Z. Performed the experiments: J.-J.S., H.-T.Y., G.-J.N., X.-W.F. and J.-F.L. Analyzed the data: J.-J.S. and J.-X.W. Wrote the paper: J.-J.S. and J.-X.W.

## Additional Information

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

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