Phosphothreonine Lyase Promotes p65 Degradation in a Mitogen-Activated Protein Kinase/Mitogen- and Stress-Activated Protein Kinase 1-Dependent Manner

Mingyu Hou,^a Wenhui Wang,^a Feizi Hu,^a Yuanxing Zhang,^{a,b,c} Dahai Yang,^{a,b} Qin Liu^{a,b,c}

^aState Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China

^bShanghai Engineering Research Center of Marine Cultured Animal Vaccines, Shanghai, China

^cShanghai Collaborative Innovation Center for Biomanufacturing, Shanghai, China

ABSTRACT Bacterial phosphothreonine lyases have been identified to be type III secretion system (T3SS) effectors that irreversibly dephosphorylate host mitogen-activated protein kinase (MAPK) signaling to promote infection. However, the effects of phosphothreonine lyase on nuclear factor κ B (NF- κ B) signaling remain largely unknown. In this study, we detected significant phosphothreonine lyase-dependent p65 degradation during *Edwardsiella piscicida* infection in macrophages, and this degradative effect was blocked by the protease inhibitor MG132. Further analysis revealed that phosphothreonine lyase promotes the dephosphorylation and ubiquitination of p65 by inhibiting the phosphorylation of mitogen- and stress-activated protein kinase-1 (MSK1) and by inhibiting the phosphorylation of extracellular signal-related kinase 1/2 (ERK1/2), p38 α , and c-Jun N-terminal kinase (JNK). Moreover, we revealed that the catalytic active site of phosphothreonine lyase plays a critical role in regulating the MAPK-MSK1-p65 signaling axis. Collectively, the mechanism described here expands our understanding of the pathogenic effector in not only regulating MAPK signaling but also regulating p65. These findings uncover a new mechanism by which pathogenic bacteria overcome host innate immunity to promote pathogenesis.

KEYWORDS MAPKs, MSK1, phosphothreonine lyase, p65 degradation

Bacterial phosphothreonine lyases are specialized type III secretion system (T3SS) effectors that specifically inactivate mitogen-activated protein kinase (MAPK) signaling pathways by removing phosphate from phosphothreonine in the pT-X-pY motif (1). Recently, a series of phosphothreonine lyases that includes OspF from *Shigella* spp., SpvC from *Salmonella* species, HopA1 from the plant pathogen *Pseudomonas syringae*, and EseH from the fish pathogen *Edwardsiella piscicida* was identified (2–8). These effectors specifically abrogate the phosphorylation and activation of extracellular signal-related kinase 1/2 (ERK1/2), p38 α , and c-Jun N-terminal kinase (JNK) signaling, thereby dampening the host immune response to bacterial infection.

In addition to the MAPK pathway, the nuclear factor κ B (NF- κ B) pathway is a central signaling cascade that is essential for the host immune response (9). Several pathogens also deliver a repertoire of T3SS effectors to interfere with NF- κ B signaling to facilitate infection. For example, the *Salmonella* T3SS effector SpvD prevents the nuclear accumulation of p65 to suppress the production of proinflammatory cytokines (10), and the protease effectors PipA, GtgA, and GogA can inhibit NF- κ B signaling by the direct degradation of p65 (11). Moreover, NleC from enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *Escherichia coli* (EHEC) is a zinc metalloprotease that suppresses NF- κ B activation by degrading p65 (12, 13). In addition to these nonphosphothreonine

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Address correspondence to Qin Liu, qinliu@ecust.edu.cn.

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lyase members, OspF was also found to effectively potentiate the degradation of I κ B α after tumor necrosis factor alpha (TNF- α) stimulation by disrupting the p38/TAK1 negative-feedback loop (14). Thus, these results suggest a bifurcated role of bacterial phosphothreonine lyases in regulating NF- κ B signaling, which needs to be further clarified.

E. piscicida is a fish pathogen with a broad host range that causes gastrointestinal septicemia in fish and wound infections in humans (15–17). Recently, we identified a T3SS effector, EseH, to be a phosphothreonine lyase that interferes with host ERK1/2, p38 α , and JNK-MAPK pathways during *E. piscicida* infection (8). This promotes *E. piscicida* virulence and colonization in a zebrafish infection model (8). Although we did not detect an effect on I κ B α degradation, whether this newly identified phosphothreonine lyase could interfere with other elements of NF- κ B signaling remains unknown. In this study, we observed significant phosphothreonine lyase-dependent p65 degradation during *E. piscicida* infection in macrophages, and we reveal that phosphothreonine lyase promotes the dephosphorylation and ubiquitination of p65 by inhibiting the phosphorylation of mitogen- and stress-activated protein kinase-1 (MSK1) in an MAPK-dependent manner. Moreover, we demonstrate that the catalytic active site of phosphothreonine lyase plays a critical role in regulating the MAPK-MSK1-p65 signaling axis. These findings suggest that the pathogenic phosphothreonine lyase can interfere not only with MAPK signaling but also with p65 signaling.

RESULTS

EseH promotes p65 degradation during *E. piscicida* infection. To investigate the effect of the newly identified phosphothreonine lyase, EseH, during *E. piscicida* infection, J774A.1 macrophages were infected with the *E. piscicida* wild-type, Δ *eseH* mutant, and Δ *eseH*::p*EseH* complemented mutant strains. First, we found that the transcription of interleukin-8 (IL-8) was significantly upregulated in J774A.1 cells infected with the Δ *eseH* mutant (Fig. 1A). Since IL-8 is an important chemokine produced by macrophages and other cell types (18) and its expression is regulated by the NF- κ B pathway (19), we stepped forward to analyze the expression level of p65 and p50, the other key subunits of the NF- κ B pathway (20). We found that the p65 abundance in J774A.1 cells was reduced in cells infected with the *E. piscicida* wild-type or Δ *eseH*::p*EseH* strain but not in those infected with the Δ *eseH* strain, while the p50 abundance was not affected during *E. piscicida* infection (Fig. 1B). These results indicate that EseH might play a critical role in p65 degradation during *E. piscicida* infection. To further investigate the role of EseH in regulating the transcription level of *p65* in this process, we found a comparable level of mRNA expression before and after *E. piscicida* infection (Fig. 1C). Moreover, we found a comparable level of *p65* mRNA expression in EseH-transfected HEK293T cells (Fig. 1D). Taken together, the results suggest that EseH-mediated p65 degradation occurs through posttranslational modification during infection.

Phosphothreonine lyases promote p65 ubiquitination-mediated degradation. To further confirm the role of phosphothreonine lyases in mediating p65 degradation, we cotransfected HEK293T cells with plasmids producing EseH-hemagglutinin (HA) and p65-Flag and observed a comparable reduced abundance of p65, indicating that EseH can promote p65 degradation in the absence of MG132 treatment (Fig. 2A). Furthermore, we evaluated the role of another phosphothreonine lyase-family protein, OspF from *Shigella*, and also detected the degradation of p65 (Fig. 2A). Since the catalytic enzyme active sites of phosphothreonine lyases are critical for regulating MAPK signaling cascades (4, 8), we cotransfected HEK293T cells with plasmids producing EseH with a K79R, H81R, or K111A mutation and p65. We found that the p65 abundance was not significantly reduced compared with that in cells transfected with wild-type EseH (Fig. 2B). Meanwhile, the indicated OspF mutants (K102R, H104A, or K134A) did not promote the degradation of p65 (Fig. 2C). Taken together, these results indicate that phosphothreonine lyases promote p65 degradation, which was dependent on their catalytic activity.

FIG 1 EseH promotes p65 degradation during *E. piscicida* infection. (A) Expression of IL-8 in J774A.1 cells infected with the *E. piscicida* wild-type, Δ eseH, and eseH-complemented strains. PBS-treated cells were used as a control. The experiments were performed in triplicate for each group. Error bars indicate the SD for technical replicates. *, $P < 0.05$. (B) Immunoblotting analysis of p65 expression. J774A.1 cells were infected with the *E. piscicida* wild-type, Δ eseH, and Δ eseH::pEseH strains at an MOI of 10 for 3.5 h. Cell lysates were probed with anti-p65, anti-p50, and anti- β -actin antibodies. All the densitometry data (presented beneath the gels) were compared to those for the control (for which the densitometry value was set equal to 100). The signal intensities were quantitatively analyzed by ImageJ software, and the data (means $\pm$ SD) are representative of those from at least three experiments. *, $P < 0.05$. (C and D) qPCR analysis of p65 expression during the indicated *E. piscicida* infection (C) or EseH transfection (D). Total RNA samples were analyzed by using the p65-specific or β -actin-specific primers. Data are representative of those from at least 3 experiments.

When detecting the expression of exogenous p65 in our transient-expression model, we conducted a parallel experiment for each group treated with MG132, a protease inhibitor (21), and found that the degradation of p65 by both EseH and OspF was impaired in the presence of MG132, which suggests that phosphothreonine lyase-mediated p65 degradation may occur through ubiquitination (Fig. 2A). To confirm this hypothesis, we cotransfected plasmids producing EseH-enhanced green fluorescent protein (EGFP) or OspF-EGFP, p65-Flag, and pRK5-HA-ubiquitin-48 into HEK293T cells, and the cells were administered MG132 to inhibit the degradation of p65. When utilizing the anti-HA-specific antibody to analyze the ubiquitination level of p65, as described in previous studies (22, 23), we found that p65 was increasingly ubiquitinated in the presence of either EseH or OspF, suggesting that phosphothreonine lyase can promote the ubiquitination of p65 (Fig. 3A and B). Furthermore, when we mutated

FIG 2 Phosphothreonine lyases promote p65 degradation. (A) HEK293T cells were cotransfected with p65-Flag and EseH-HA or OspF-HA with or without MG132. Cell lysates were collected and probed for anti-Flag, anti-HA, and anti- β -actin antibodies. (B) HEK293T cells were cotransfected with p65-Flag and EseH-HA or the indicated mutants. Cell lysates were collected and probed for anti-Flag, anti-HA, and anti- β -actin antibodies. (C) HEK293T cells were cotransfected with p65-Flag and OspF-HA or the indicated mutants. Cell lysates were collected and probed for anti-Flag, anti-HA, and anti- β -actin antibodies. All the densitometry data (presented beneath the gels) were compared to those for the control (for which the densitometry value was set equal to 100). The data (means $\pm$ SD) are representative of those from at least three experiments. The signal intensities were quantitatively analyzed by ImageJ software. *, $P < 0.05$.

either EseH or OspF at their enzyme catalytic active sites, as in the assay whose results are presented in Fig. 2, the ubiquitinated p65 was comparatively reduced (Fig. 3A and B). Thus, these results suggest that the bacterial phosphothreonine lyase may play an important role in promoting the ubiquitination of p65 to mediate its degradation.

Phosphothreonine lyase inhibits p65 phosphorylation by inhibiting MAPK-MSK1 axis activation. When the effectors are delivered into host cells during bacterial infection, MAPKs are the direct targets of phosphothreonine lyases (2–8). MSK1 is a kinase downstream of MAPKs (24) and is directly activated by ERK1/2 and p38 α by phosphorylation at Ser360 (25), which is responsible for p65 phosphorylation to maintain its stability (26). To confirm that the activation of MSK1 is regulated by the activation of MAPK, we treated the cells with specific inhibitors (27–29). When we treated the cells with U0126 and SB208530, inhibitors of ERK1/2 and p38, respectively, we consistently found a significant inhibition of the phosphorylation of MSK1 (Fig. 4A and B). Interestingly, we also observed a significant inhibition of MSK1 phosphorylation following treatment with SP600125, a specific JNK inhibitor (Fig. 4C). Taken together, our results indicate that the phosphorylation of MSK1 can be inhibited by the inhibition of MAPK activation.

Based on the results presented above, we hypothesized that the phosphothreonine lyase-mediated p65 ubiquitination and degradation occur through the MAPK-MSK1 signaling pathway. To confirm this, we first verified the phosphorylation of ERK1/2, p38 α , and JNK in macrophages during *E. piscicida* infection. Consistent with previous results (8), EseH could inhibit the phosphorylation of these MAPKs (Fig. 4D), and the

FIG 3 Phosphothreonine lyases promote p65 ubiquitination. (A) HEK293T cells were cotransfected with p65-Flag, ubiquitin-HA, and the indicated EseH. MG132 was added to protect the exogenous p65 from degradation. Cell lysates were immunoprecipitated (IP) with anti-Flag beads and then analyzed by immunoblotting with anti-HA, anti-Flag, or anti-EGFP antibodies. (B) HEK293T cells were cotransfected with p65-Flag, ubiquitin (Ubi)-HA, and the indicated OspF. MG132 was added to protect the exogenous p65 from degradation. Cell lysates were immunoprecipitated with anti-Flag beads and then analyzed by immunoblotting with anti-HA, anti-Flag, or anti-EGFP antibodies. The data (means $\pm$ SD) are representative of those from at least three experiments. The signal intensities were quantitatively analyzed by NIH ImageJ software.

enzyme catalytic active site was critical for regulating this process (Fig. 4E). Thus, we analyzed whether phosphothreonine lyase inhibited the phosphorylation of MSK1. We found that the phosphorylation of MSK1 was inhibited upon infection with the *E. piscicida* wild-type or Δ *EseH*::*pEseH* strain but not with the Δ *EseH* mutant (Fig. 4D). To analyze whether phosphothreonine lyase can directly affect the phosphorylation of MSK1, we cotransfected HEK293T cells with plasmids producing EseH and its mutants (the K79R, H81R, or K111A mutant) and then administered TNF- α to the cells. We found that the phosphorylation of MSK1 was inhibited by EseH but not by the indicated mutants (Fig. 4E). Consistently, OspF, but not its mutants, could also inhibit the phosphorylation of MSK1 (Fig. 4F). Taken together, these results suggest that phosphothreonine lyase can inhibit the phosphorylation of MSK1.

Phosphothreonine lyase inhibits p65 phosphorylation to promote its ubiquitination. The phosphorylation of MSK1 leads to the direct phosphorylation of p65 at Ser276, thereby preventing p65 from degradation (30). Moreover, the p65 S276A mutant is degraded by the ubiquitin-proteasome machinery (31). Thus, to further test the hypothesis that phosphothreonine lyase-mediated p65 degradation is associated with the dephosphorylation and ubiquitination of p65, we first evaluated whether the inactive MAPKs could inhibit p65 phosphorylation. We first analyzed the phosphorylation of MSK1 during MAPK- and MSK1-specific inhibitor treatment. Cells were treated with MG132 to protect against p65 degradation. We found that the p65 abundance in each group remained the same after treatment, but the phosphorylation levels of p65 at Ser276 were significantly reduced upon treatment with the inhibitors indicated in Fig. 5A. Next, we investigated the phosphorylation of p65 during *E. piscicida* infection in J774A.1 cells treated with MG132, to inhibit p65 degradation, and analyzed the phosphorylation of p65. The results revealed that the phosphorylation of p65 was reduced in cells infected with the *E. piscicida* wild-type or Δ *EseH*::*pEseH* strain but not the Δ *EseH* mutant, which revealed that the phosphorylation of p65 is inhibited by EseH during *E. piscicida* infection (Fig. 5B).

In addition, to analyze the role of p65 Ser276 in regulating the ubiquitination of p65, we cotransfected HEK293T cells with plasmids expressing EGFP or EseH-EGFP and plasmids expressing p65-Flag or p65 (S276A)-Flag with pRK5-HA-ubiquitin-48 express-

FIG 4 Phosphothreonine lyases inhibit the phosphorylation of MSK1. (A to C) J774A.1 cells were pretreated with 10 μ M U0126 (A), 10 μ M SB208530 (B), or 10 μ M SP600125 (C), and then TNF- α was administered for 2 h. Cell lysates were probed with anti-phospho-ERK1/2, anti-phospho-p38 α , anti-phospho-JNK, anti-phospho-MSK1 (Ser360), and anti-MSK1. β -Actin was probed as a control. (D) J774A.1 cells were infected with the *E. piscicida* wild-type, Δ *eseH*, and Δ *eseH::pEseH* strains at an MOI of 10 for 3.5 h. Cell lysates were collected and probed with anti-phospho-MSK1 (Ser360), anti-MSK1, anti-phospho-ERK1/2, anti-p38 α , anti-phospho-JNK, and anti- β -actin antibodies. (E and F) HEK293T cells were cotransfected with the indicated EseH-HA (E) or the indicated OspF-HA (F), and then the cells were treated with TNF- α for 2 h. Cell lysates were collected and probed with anti-MSK1, anti-phospho-MSK1, anti-HA, anti- β -actin, anti-phospho-ERK1/2, anti-phospho-p38 α , and anti-phospho-JNK antibodies. All the densitometry data (presented beneath the gels) were compared to those for the control (for which the densitometry value was set equal to 100). The data (mean $\pm$ SD) are representative of those from at least three experiments. The signal intensities were quantitatively analyzed by ImageJ software. *, $P < 0.05$.

ing ubiquitin-HA. We found that p65 with the S276A mutation was more highly ubiquitinated than wild-type p65, and this ubiquitination was increased in the presence of EseH (Fig. 5C), which suggests that the phosphorylation of Ser276 is critical for preventing p65 from ubiquitination. Taken together, these results indicate that the

FIG 5 Phosphothreonine lyase inhibits the phosphorylation of p65 at Ser276 residues to promote its ubiquitination. (A) J774A.1 cells were pretreated with U0126 (10 μ M), SB208530 (10 μ M), SP600125 (10 μ M), or H89 (50 μ M) and then infected with *E. piscicida* Δ *eseH* at an MOI of 10 for 3.5 h. Cell lysates were probed with anti-phospho-p65 (Ser276), anti-p65, and anti- β -actin antibodies. (B) J774A.1 cells were infected with the *E. piscicida* wild-type, Δ *eseH*, or Δ *eseH::pEseH* strain at an MOI of 10 for 3.5 h. MG132 was added to inhibit the degradation of p65. Cell lysates were collected and probed with anti-phospho-p65, anti-p65, and anti- β -actin antibodies. (C) HEK293T cells were cotransfected with p65- or p65 (S276)-Flag, EseH-EGFP, and ubiquitin-HA. MG132 was added to inhibit the degradation of p65. Cell lysates were immunoprecipitated with anti-Flag beads and then analyzed by immunoblotting with anti-HA, anti-Flag, or anti-EGFP antibodies. All the densitometry data (presented beneath the gels) were compared to those for the control (for which the densitometry value was set equal to 100). The data (mean $\pm$ SD) are representative of those from at least three experiments. The signal intensities were quantitatively analyzed by NIH ImageJ software. *, $P < 0.05$.

bacterial phosphothreonine lyase can promote p65 ubiquitination through the inhibition of Ser276 phosphorylation.

DISCUSSION

As p65 is an important component of the NF- κ B signaling pathway, pathogens have evolved multiple strategies to subvert this protein (10–13). Previous studies have proved that the phosphothreonine lyase family of proteins can directly inhibit MAPK pathway activation (4, 8) and that MAPK signaling regulates the phosphorylation of downstream MSK1 (32, 33). MSK1 plays a critical role in regulating the phosphorylation of p65 at Ser276, keeping the stability of p65 (30, 34). These signaling cascades connect the MAPK pathway and the NF- κ B pathway, which prompted us to analyze the role of bacterial phosphothreonine lyases during infection. A previous study revealed that OspF can interact with HP1 γ and alter its phosphorylation at Ser83 by inactivating ERK and, consequently, MSK1 (35). However, the downstream consequences of phosphothreonine lyase acting on NF- κ B signaling were unknown. In this study, we revealed the role of the bacterial phosphothreonine lyases EseH and OspF in regulating the MAPK-MSK1 axis and clarified their critical role in promoting p65 degradation (Fig. 6). Interestingly, we found that either during bacterial infection or through the use of pathway inhibitors, JNK-MAPK signaling plays a role in regulating MSK1 phosphorylation, which is different from the effect of OspF on the phosphorylation of HP1 γ (35). We found that EseH and OspF dephosphorylate p65 through their lyase activity on ERK, p38, and JNK. Another interesting phenomenon is that we found that EseH can promote the more serious ubiquitination of p65 (S276A) than that of wild-type p65 (Fig. 5C), which indicates that there might be some interesting sites on p65 which could be

FIG 6 Summary of proposed mechanism for the bacterial phosphothreonine lyase. Phosphothreonine lyase targets MAPKs to dephosphorylate MSK1 and p65 and promotes the ubiquitination of p65, which results in the degradation of p65.

ubiquitinated by phosphothreonine lyases. Collectively, although our results extend the function of bacterial EseH and OspF during infection, whether other phosphothreonine lyase-family proteins share the same effects on this pathway remains to be clarified.

Although EseH is not the first effector identified in *E. piscicida*, the present work is the first to explain how a T3SS effector of *E. piscicida* interferes with the host NF- κ B pathway. In our previous study, we detected the degradation of I κ B during *E. piscicida* infection in HeLa cells and found that there is no significant difference between wild-type and Δ *eeseH* mutant infection (8). However, both I κ B and p65 are important elements of the NF- κ B pathway and remain attractive targets for exploitation by microbial pathogens to modulate host cell events (36). In this study, we analyzed the mechanism of phosphothreonine lyase in regulating p65 degradation during *E. piscicida* infection in macrophages, revealing the role of this important bacterial T3SS effector not only in targeting MAPKs but also in regulating NF- κ B, thereby uncovering a new mechanism by which pathogenic bacteria overcome host innate immunity to promote pathogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5 α was used for recombinant DNA experiments. *Escherichia coli* cells were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. *Edwardsiella piscicida* and derivative strains were grown in Trypticase soy broth or tryptic soy agar at 30°C. When necessary, ampicillin (100 μ g/ml) and colistin (16.7 μ g/ml) were added to the media.

Cell cultures, infection, and transfection. The murine macrophage-like cell line J774A.1 (ATCC TIB-67; ATCC, Manassas, VA, USA) and human embryonic kidney HEK293T cells (ATCC CRL-3216) were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C with 5% CO₂. J774A.1 cells were infected at a multiplicity of infection (MOI) of 10:1 in DMEM at 35°C for 3.5 h. HEK293T cells were transfected using the standard calcium phosphate method.

Construction of plasmids. Primers EseH-EGFP-F and EseH-EGFP-R were used to prepare EseH-EGFP and its mutants containing an EGFP tag. The DNA sequence of the OspF gene was synthesized by the Genexiz company (Suzhou, China). Primers OpsF (K102R)-F and OpsF (K102R)-R, OpsF (H104A)-F and OpsF (H104A)-R, and OpsF (K134A)-F and OpsF (K134A)-R were used to insert OspF mutants in pairs. To add pCDH homologous flanks and an HA tag or EGFP tag, primers OspF-HA-F and OspF-HA-R or OspF-EGFP-R were used for an additional amplification using OspF and its mutants as models. In parallel, total cellular RNA was extracted from J774A.1 cells using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA was reverse transcribed to cDNA using a FastKing

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Strains		
<i>Escherichia coli</i> DH5 α	$\Delta(lacZYA-argF)U169 \phi 80dlacZ\Delta M15$	Lab collection
<i>Edwardsiella piscicida</i>		
EIB202	<i>Edwardsiella piscicida</i> wild-type strain, Col ^r Cm ^r	CCTCC no. M208068
Δ <i>eseH</i>	EIB202 with deletion of <i>eseH</i>	8
Δ <i>eseH::pEseH</i>	The Δ <i>eseH</i> strain complemented with pUTt-0456-EseH-HA, Amp ^r	8
Plasmids		
pCDH-HA	pCDH-CMV-MCS derivative containing HA tag	8
pCDH-EseH-HA	pCDH-CMV-MCS derivative containing EseH-HA fusion	8
pCDH-EseH (K79R)-HA	pCDH-CMV-MCS derivative containing EseH(K79R)-HA fusion	8
pCDH-EseH (H81R)-HA	pCDH-CMV-MCS derivative containing EseH(H81R)-HA fusion	8
pCDH-EseH (K111A)-HA	pCDH-CMV-MCS derivative containing EseH(K111A)-HA fusion	8
pCDH-OspF-HA	pCDH-CMV-MCS derivative containing OspF-HA fusion	This study
pCDH-OspF (K102R)-HA	pCDH-CMV-MCS derivative containing OspF (K102R)-HA fusion	This study
pCDH-OspF (H104R)-HA	pCDH-CMV-MCS derivative containing OspF (H104R)-HA fusion	This study
pCDH-OspF(K134A)-HA	pCDH-CMV-MCS derivative containing OspF(K134A)-HA fusion	This study
pCDH-EGFP	pCDH-CMV-MCS derivative containing EGFP tag	This study
pCDH-EseH-EGFP	pCDH-CMV-MCS derivative containing EseH-EGFP fusion	This study
pCDH-EseH (K79R)-EGFP	pCDH-CMV-MCS derivative containing EseH(K79R)-EGFP fusion	This study
pCDH-EseH (H81R)-EGFP	pCDH-CMV-MCS derivative containing EseH(H81R)-EGFP fusion	This study
pCDH-EseH (K111A)-EGFP	pCDH-CMV-MCS derivative containing EseH(K111A)-EGFP fusion	This study
pCDH-OspF-EGFP	pCDH-CMV-MCS derivative containing OspF-EGFP fusion	This study
pCDH-OspF (K102R)-EGFP	pCDH-CMV-MCS derivative containing OspF (K102R)-EGFP fusion	This study
pCDH-OspF (H104R)-EGFP	pCDH-CMV-MCS derivative containing OspF (H104R)-EGFP fusion	This study
pCDH-OspF (K134A)-EGFP	pCDH-CMV-MCS derivative containing OspF (K134A)-EGFP fusion	This study
pCDH-p65-Flag	pCDH-CMV-MCS derivative containing p65-Flag fusion	This study
pCDH-p65 (S276A)-Flag	pCDH-CMV-MCS derivative containing p65 (S276A)-Flag fusion	This study
pRK5-HA-ubiquitin-48	Mammalian expression of HA-tagged ubiquitin with only K48, Amp ^r	Addgene

one-step reverse transcription (RT)-PCR kit (Tiangen, Beijing, China), and p65 was amplified from total cDNA using primers mp65-F and mp65-R. The PCR product was used as a model to amplify the p65-Flag fragment or p65 (S276A)-Flag containing ends compatible with pCDH. The primers used are listed in Table 2. All fragments were inserted into the linearized pCDH plasmid using a one-step cloning kit (Vazyme Biotech, Jiangsu, China).

TABLE 2 Primers used in this study

Primer name	Primer sequence (5' to 3')
IL-8 (RT)-F	TCGAGACCATTTACTGCAACAG
IL-8 (RT)-R	CATTGCCGGTGGAAATTCCTT
p65 (RT)-F	TGCGATTCCGCTATAAATGCG
p65 (RT)-R	ACAAGTTCTATGTGGATGAGGC
β -actin (RT)-F	GTGACGTTGACATCCGTAAAGA
β -actin (RT)-R	GCCGGACTCATCGTACTCC
p65-F	ATGGACGATCTGTTTCCCTC
p65-R	TTAGGAGCTGATCTGACTC
Flag-p65-F	CCATAGAAGATATGGACGATCTG
Flag-p65-R	GATTTAAATTCCTTACTTGTCGTCATCGTCTTTGTAGTCGGAGCTGATC
p65 (S276A)-R1	CGCGATCAGCAGGCCGC
p65 (S276A)-F2	GCGGCCTGCTGATCGCG
OspF-HA-F	CCATAGAAGATATGCCATAAAAAAGCCCTGTC
OspF-HA-R	GATTTAAATTCCTAGGCATAGTCTGGGACGTCA
OspF(K102R)-F	GGGGACAGGTTTCATATTAG
OspF(K102R)-R	CTAATATGAAACCTGTCCCC
OspF(H104A)-F	GACAAGTTTGCTATTAGTA
OspF(H104A)-R	TACTAATAGCAAACCTGTGTC
OspF(K134A)-F	GATAAATGGGCGATAACTG
OspF(K134A)-R	CAGTTATCGCCCATTTATC
OspF-EGFP-R	CTCGCCCTTGCTCACGGCATAGTC
EseH-EGFP-F	CTGTTTTGTCTAGAATGCCCC
EseH-EGFP-R	CCTTGCTCACGCTAGCCTCTGT
line-pCDH-F	ATCTTCTATGGAGGTCAAAACAGC
line-pCDH-R	GAATTCGAATTTAAATCGGATCCG
line-pCDH-EGFP-F	GTGAGCAAGGGCGAG

RNA extraction and quantitative real-time PCR. Total RNA was extracted from *E. piscicida* wild-type-, *DeseH* strain-, and *DeseH::pEseH* strain-infected J774A.1 cells. The TRIzol reagent (1 ml; Invitrogen) was used for each well, and cDNA was generated using a FastKing RT kit (Tiangen). Real-time quantitative PCR (qPCR) was performed with Super Real premix (Tiangen) in eight-well strip tubes. The gene for β -actin was used as a reference to obtain the relative fold changes in expression in the target samples by the comparative $\Delta\Delta C_T$ threshold cycle (C_T) method. Primers were designed with the Integrated DNA Technologies Primer Quest tool. The primers used are listed in Table 2 and were IL-8 (RT)-F, IL-8 (RT)-R, β -actin (RT)-F, β -actin (RT)-R, p65 (RT)-F, and p65 (RT)-R. RT-qPCR experiments were carried out in 3 biological replicates per strain, and each condition and all qPCRs were performed in three biological replicates.

Ubiquitination detection assay. Eukaryotic expression vectors pRK5-HA-ubiquitin-48 and pCDH-p65-Flag or pCDH-p65 (S276A)-Flag were transfected with pCDH-EseH-EGFP (or its mutants) or pCDH-OspF-EGFP (or its mutants), and MG132 was added to protect against p65 degradation. At 24 h after transfection, 1 ml lysis buffer (20 mM Tris, 100 mM KCl, 1 mM EDTA, 10 mM $\text{Na}_2\text{P}_2\text{O}_7$, 10% glycerol, 0.1% NP-40, pH 7.5) was used to lyse the cells in a 10-cm dish, and the supernatants were subjected to immunoprecipitation with anti-Flag M2 magnetic beads (Sigma, St. Louis, MO, USA). Immunoblotting was used to detect ubiquitin with an HA antibody (HuaAN Biotechnology, Oxfordshire, UK).

Pathway inactivation. For pathway inactivation experiments, 10 μM U0126 (Selleck Chemicals, Houston, TX, USA) was used to inactivate the ERK1/2 pathway, 10 μM SB208530 (Selleck Chemicals) was used to inactivate the p38 α pathway, 10 μM SP600125 (Selleck Chemicals) was used to inactivate the JNK pathway, and 50 μM H89 (MedChemExpress, Monmouth Junction, NJ, USA) was used to inactivate the MSK1 pathway. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) and added to the cell culture media for 2 h, while the same volume of DMSO alone was added as a control for the same amount of time. The 26S proteasome inhibitor MG132 (Selleck Chemicals) was dissolved in phosphate-buffered saline (PBS) and used at a concentration of 10 μM .

Western blotting and antibodies. Harvested cells were lysed in buffer containing 1% Triton X-100, 50 mM Tris, 150 mM NaCl, and 1 mM EDTA (pH 7.4). Lysates were mixed with protein loading buffer, boiled, and centrifuged. Samples were then subjected to SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) using a standard electroblotting system. After blocking with 5% bovine serum albumin plus 0.05% PBS-Tween 20 (PBST), the membranes were incubated at room temperature for 2 h with primary antibodies. The phospho-ERK1/2 antibody (catalog number 9101; CST), phospho-p38 α antibody (catalog number 9215; CST), phospho-JNK antibody (catalog number 9251; CST), anti-MSK1 (phosphor-S360) antibody (catalog number ab81294; Abcam), p65 (phosphor-S276) antibody (catalog number ab106129; Abcam), MSK1 antibody (catalog number AF2518; R&D Systems), EGFP antibody (catalog number MAB4401; Tiangen), HA antibody (catalog number 0906; HuaAN), Flag antibody (catalog number 0912; HuaAN), p65 antibody (catalog number ET1603-12; HuaAN), β -actin antibody (catalog number M1210-2; HuaAN), and p50 antibody (catalog number AF1246; Beyotime) were probed. Next, the membranes were washed with PBST and incubated with a 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibody (Beyotime) at room temperature for 2 h.

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D.Y., Q.L., and M.H. conceived the study; M.H. performed most of the experiments. W.W. and F.H. helped with plasmid construction. Y.Z. provided expert advice and critically reviewed the manuscript. The manuscript was written by M.H. and D.Y. Q.L. supervised the study. All authors discussed the results and commented on the manuscript.

We declare that there are no conflicts of interest.

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