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POSH is involved in Eiger-Basket (TNF-JNK) signaling and embryogenesis in *Drosophila*

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Abstract

TNFα can trigger different signaling pathways, including the JNK pathway, to regulate various biological functions such as cell death, differentiation and proliferation. The scaffold protein POSH (Plenty of SH3 Domains) has been shown to be an important regulator of the JNK pathway, but whether it is involved in TNF-signaling has not been reported. Although POSH has been implicated to play a role in development in zebrafish, it has not been studied in null mutants and the underlying mechanism of its effects is still not clear. In this study, we provide evidence that the JNK pathway scaffold protein, POSH, is involved in TNF (Eiger) signaling in *Drosophila*. POSH is likely to act downstream of dTAB2 and upstream of dTAK1 in the TNF-JNK signaling pathway. In addition, we found that *POSH* is essential during *Drosophila* embryogenesis, including epidermal dorsal closure, similar to other JNK pathway components such as *Silpper*, *Hemipterous*, and *Basket*. We observed defects in F-actin accumulation and adherens junction formation during dorsal closure in different *posh* null mutants, suggesting that POSH is required for epidermal cell migration and cell-shape change during epidermal dorsal closure.

Keywords: TNF; JNK; POSH; embryogenesis; cell migration; adherents junction

Introduction

Activation of the TNFR (Tumor Necrosis Factor Receptor) family by tumor necrosis factors (TNFs) plays important roles in various fundamental processes in mammals including apoptosis, cell survival, differentiation, proliferation, and inflammation. Deregulation of the TNF signaling pathway is associated with a wide spectrum of diseases, including immune disorders, cancer and developmental defects (Bossen et al., 2006). The binding of TNF to its receptor leads to the formation of different protein complexes that regulate the NF-κB (Nuclear Factor-κB) or the JNK (c-Jun N-terminal Kinase) pathways, which in turn influence cell-death and cell-survival decisions. Similar to mammalian TNF, the *Drosophila* TNF homolog Eiger (Egr) triggers cell death through JNK signaling (Igaki et al., 2002). The signaling transduction of the TNF-JNK pathway involves the receptor Wengen (Wgn), which activates the JNK cascade, involving a MAP kinase kinase kinase (MAPKKK, dTAK1), a MAP kinase kinase (MAPKK, Hep), and JNK (Bsk). In addition, other important components of the TNF-signaling pathway involved in the regulation of the NF-κB pathway, such as TNF receptor associated factors (TRAFs) and TAK1-associated binding protein 2 (TAB2), are also conserved in *Drosophila* (Igaki

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et al., 2002; Moreno et al., 2002; Geuking et al., 2005).

Scaffold proteins are recognized as critical regulators in promoting signal transduction activity and specificity by the assembly of multi-protein signaling complexes (Morrison and Davis, 2003; Dhanasekaran et al., 2007). Two distinct families of scaffold proteins in the JNK pathway, the JIPs (JNK Interacting Proteins) and POSH (Plenty of SH3 Domains) have been well studied (Morrison and Davis, 2003; Xu et al., 2003). POSH was originally identified as a binding partner of the activated form of Rac1 and a potential activator of both NF-κB and JNK signaling (Tapon et al., 1998). We have previously demonstrated that POSH serves as a scaffold protein for the JNK cascade and plays a required role in neuronal apoptosis (Xu et al., 2003). Our subsequent studies indicated that POSH coordinates with JIP in organizing different components of the JNK pathway, including Rac1, mixed lineage kinases (MLKs, a MAP3K family), MKK4, MKK7 and JNKs into a complex named as the POSH-JIP apoptotic complex (PJAC) (Kukekov et al., 2006). Under apoptotic conditions, stabilization of POSH, JIPs and MLKs through JNK-induced phosphorylation occurs, and facilitates a 'feed-forward' loop. This positive feedback mechanism leads to the activation of JNK to reach a threshold that induces cell death (Xu et al., 2005).

In addition to its role in apoptosis, POSH has been shown to be involved in other biological processes by acting as an E3 ligase to mediate protein degradation. For example, POSH interacts with other proteins such as Hrs (hepatocyte growth factor-regulated tyrosine substrate) to regulate endosome sorting and the production of infectious HIV-1 (Alroy et al., 2005; Votteler et al., 2009), HERP (Homocysteine-inducible Endoplasmic Reticulum Protein) to regulate calcium homeostasis (Tuvia et al., 2007), TAK-1 (TGF-beta-Activated Kinase-1) to regulate immune response (Tsuda et al., 2005), ALIX (ALG2 Interacting protein X) to regulate virus release (Votteler et al., 2009) and POSH itself to regulate JNK signaling (Xu et al., 2003).

POSH is evolutionarily conserved from *Drosophila* to human. They share a very similar structure containing a ring-finger domain and four SH3 domains, except for the lack of the typical Rac-binding motif in *Drosophila*. In *Drosophila*, over-expression of *POSH* in neural tissues extends the longevity of adult flies without affecting viability or morphology, while over-expression of *POSH* in non-neuronal tissues induces JNK signaling and the cell death process leading to morphological defects, such as loss of cross-veins, notched wings, and disordered hair polarity (Seong et al., 2001a, 2001b). The morphological defects in wings can be rescued by *puc*, *bsk*, *or hep*, suggesting a role of POSH in the JNK pathway. It has been reported recently that a null allele of *posh* was viable but displayed innate immune system defects likely due to the deregulation of JNK and NF-κB signaling (Tsuda et al., 2005). A genetic screen for regulators of *Drosophila* egg shell structure found that a hypomorph allele of *POSH* is a weak enhancer of *Ras1* in embryogenesis (Schnorr et al., 2001). Knockdown of *POSH* expression in *Xenopus* causes anterior truncations and optical developmental defects (Kim et al., 2005).

Although previous studies indicate that POSH is a critical regulator of the JNK pathway *in vitro*, whether it is involved in TNF-signaling has not been reported. In addition, the role of POSH in development in null mutants and the underlying molecular mechanisms remain to be elucidated. We show evidence here that the scaffold protein POSH plays an important role in the TNF-signaling pathway *in vivo*. By generating and analyzing of *posh* complete null mutants, we found that *POSH* is essential during development and is required for morphogenesis, including dorsal closure, embryonic head involution and adult thorax closure. Our results suggest that the dorsal closure defect of *posh* mutants is very likely due to the deregulation of the cytoskeleton and adherens junctions, leading to the failure of epidermal cell elongation and cell migration during dorsal closure.

Materials and methods

Drosophila strains

The following fly stocks were used: *UAS-POSH* II (from Dr. T. Aigaki); *UAS-POSH*^{RNAi} II (VDRC#26657); *posh*^{k15815}*/CyO* (BL#11146); *posh*^{EP1206}*/CyO* (BL#16697); *posh* mutants *posh*^{p92} and *posh*^{p327} generated by imprecise excision of *posh*^{EP1206}; *yw; posh*^{74} (from Dr. T. Aigaki); *yw*, *dtak1*; *UAS-Egr/MRKS*; *UAS-dTAB2/CyO*; *UAS- dTraf1-IR* (from L. Xue lab); *Df(2R)BSC45/SM6a* (BL #7441); *Actin5C-Gal4/TM3*; *Pannier-Gal4/TM6B*; *Armadillo-Gal4/TM6B*; *Engrailed-Gal4/CyO; Tubulin-Gal4/TM6B*; *GMR-Gal4/CyO*; *Ap-Gal4/Cyo*; *puc*^{E69}*/TM3*; *bsk*^{2}*/CyO*; *w**, *hep*^{r75}*/FM7c*, *w**; *L*^{2} *Pin*^{1}*/Cyo, Kr:GFP* and *yw* (from Z. Wang lab). *posh*^{k15815}, *posh*^{p92} and *posh*^{p327} were balanced by *w**;

L^2 Pin^1/Cyo, Kr:GFP and homozygous mutants were identified as animals without GFP markers. All fly crosses and egg collections were performed at 25°C on standard fly media.

Generation of posh deletion mutants

posh loss-of-function mutants were generated through imprecise P-element excision of *posh^{EP1206}* using a standard protocol. Sequence analysis of *posh* mutants was based on FlyBase Release 4.2 annotations. The Gene ID for POSH is FBgn0040294. The primers used for PCR and sequencing were: 5′-GACTCCCTTGAGACCG-3′ and 5′-GCTTTACTACACCGACT-3′.

Western blotting

Embryos without GFP signal were selected as *posh* homozygous mutants for Western blotting. Two hundred stage 13–17 embryos or two adults were homogenized in 20 μL Cell Lysis Buffer (0.15 mol/L NaCl, 5mmol/L EDTA, pH 8, 1% Triton X-100, 10 mmol/L Tris-Cl, pH 7.4, 0.5 mmol/L DTT, 0.1 mmol/L PMSF) on ice, and centrifuged at 12,000 r/min for 15 min. Protein samples were prepared and analyzed as described previously (Xu et al., 2003) and were probed with anti-Posh antibody (generated against the C-terminus of POSH, a gift from Dr. T. Aigaki, 1:100) (Tsuda et al., 2005) or anti-Tubulin antibody (Sigma, USA, 1:2,000) as loading controls.

Immunostaining

Embryos were collected at different stages, dechorionated, and fixed with 4% formaldehyde in PBS with 0.1% Triton for 20 min. Antibodies used were: rabbit anti-Posh (a gift from Dr. T. Aigaki, 1:20) (Tsuda et al., 2005); monoclonal anti-phosphor-Tyrosine (Cell Signaling, USA, 1:100); rat anti-DECad, DCAD2 (DHSB, USA, 1:50); MAb anti-Dlg (DHSB; 1:100). F-actin in embryos was labeled with Alexa568-Phalloidin (Molecular Probe, USA, 2U per experiment). All fluorophore-conjugated secondary antibodies were used at 1:2,000 (Invitrogen, USA).

Embryo staging

To ensure the embryos selected were at the right stage, flies laid eggs on apple juice agar plates for 1 h, which were left to develop at 25°C. Late stage 13, stage 14, 15 and 16–17 embryos were collected after growing on juice plates for about 10, 11, 12 and 13–21 h, respectively.

Cuticle preparation

Embryos were collected, dechorionated in 50% bleach for 2 min, washed with water, vigorously shaken in heptone:formaldehyde (1:1) for 1–2 min and then left at room temperature for 30 min. Embryo cuticles were expanded and mounted, and placed at 65°C overnight for cuticle phenotype checking. Before immunostaining, embryos were washed in PBS with 0.1% Triton and blocked in 10% blocking buffer.

Imaging

Dark field images were collected under a Leica S8APO microscope for embryo cuticle phenotyping. Immunostaining images were collected with a Leica TCS SP5 confocal laser scanning microscope. Multiple optical sections were scanned. Image J and Leica LAS AF software was used for image processing.

Results

POSH is involved in the TNF-JNK signaling pathway

In *Drosophila*, the homolog of TNF, Eiger, can induce cell death as in mammals (Igaki et al., 2002; Moreno et al., 2002) and POSH has been shown to interact with dTAK1 (Tsuda et al., 2005). However, whether POSH is involved in the TNF-JNK signaling pathway is still not clear.

To examine the role of POSH in the TNF-JNK pathway, we tested the genetic interactions between POSH and Eiger (Egr). In *Drosophila*, *eiger* mutants have been reported to be viable (Igaki et al., 2002). However, ectopic expression of *Egr* under the control of *Pannier-Gal4* (*Pnr-Gal4*) induces JNK activation and causes pupal stage lethality (Igaki et al., 2002; Moreno et al., 2002). While animals with knockdown of *POSH* expression by RNAi under the control of *Pnr-Gal4* can survive to adulthood, they display thorax closure defects (Fig. 1B). The partial suppression of the pupal lethality *Pnr>Egr* phenotype by RNAi-mediated down-regulation of *POSH* (Fig. 1E) indicates that POSH may mediate Egr signaling in *Drosophila*.

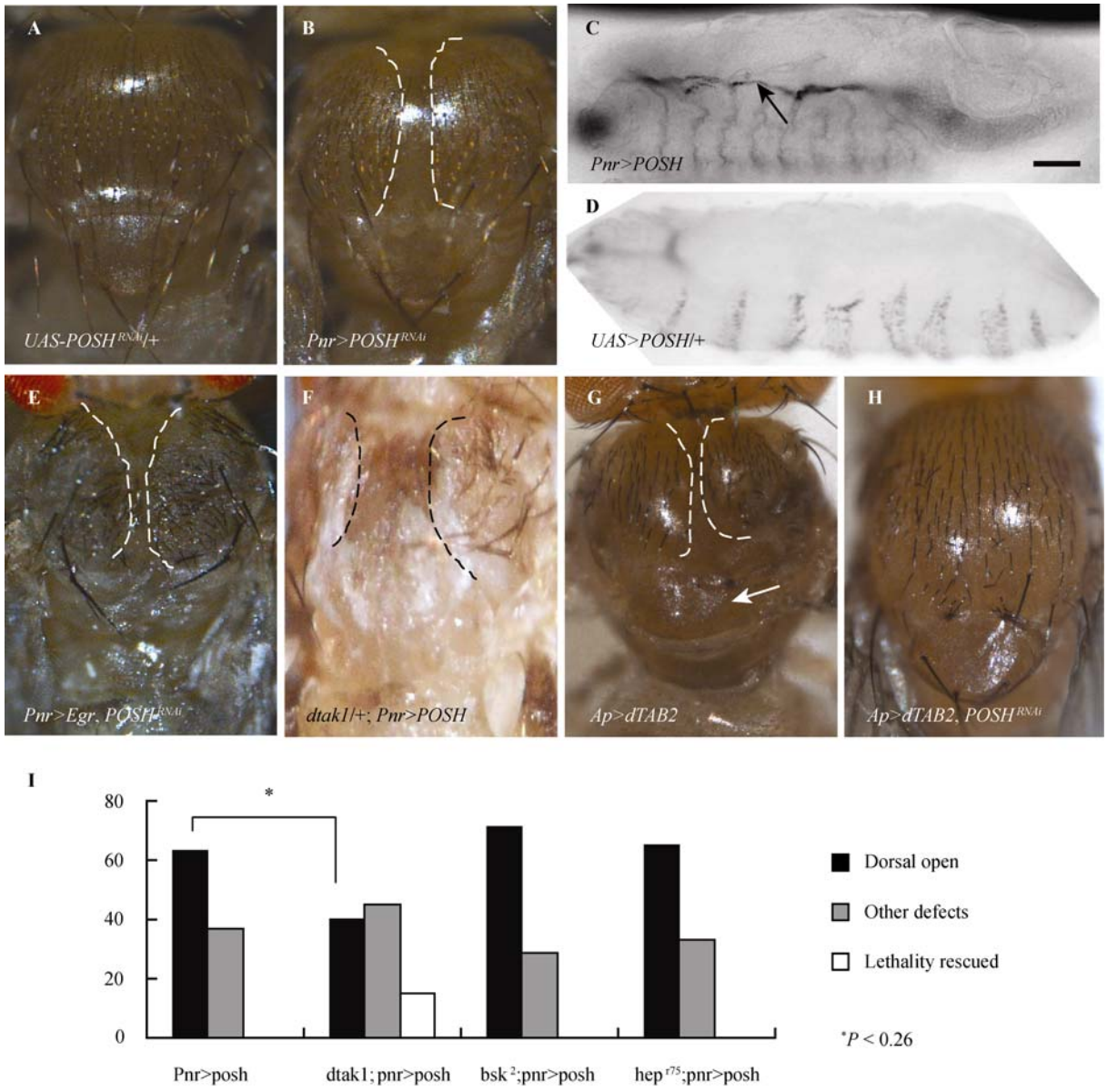

Fig. 1. POSH interacts genetically with Eiger, dTAB2 and dTAK1 during development. **A:** adult thorax of *UAS-POSH* ^{RNAi}/+ as wild-type control. **B:** knockdown of *POSH* expression by RNAi developed thorax cleft. **C** and **D:** animals over-expressing *POSH* under the control of *Pnr-Gal4* died at the embryo stage with severe dorsal closure defects (arrow in **C**), compared to wild-type **(D)**. **E:** *POSH* expression reduced by RNAi partially rescued the lethal phenotype in *Pnr>Eiger* pupa. The flies survived to adulthood with a thorax cleft. **F:** reducing the *dTAK1* dosage by half in *Pnr>POSH* suppressed the lethal dorsal open phenotype. Escapees survived to adulthood with thorax defects. **G:** over-expression of *dTAB2* driven by *Ap-Gal4* caused defects in thorax development including a thorax cleft, short scutellum and lack of bristles. **H:** knockdown of *POSH* expression rescued the thorax developmental defects of *Ap>dTAB2*. **I:** genetic interaction of *Pnr>POSH* with components of JNK pathway *dTAK1*, *bsk* ^{2} and *hep* ^{r75}. Downregulation of dTAK1 (JNKKK) partially rescues the *Pnr>POSH* induced dorsal closure defect ($P < 0.26$), while deletion of one copy of either bsk (JNK) or hep (JNKK) has no effect. *P* values reflecting significances among the data were confirmed by conducting a *chi*-squared test. Genotype: **A**, *UAS-POSH* ^{RNAi}/+; **B**, *UAS-POSH* ^{RNAi}/+;*Pnr-Gal4*/+; **C**, *UAS-POSH*/+;*Pnr-Gal4*/+, embryo stage; **D**, *UAS-POSH*/+, embryo stage; **E**, *UAS-POSH* ^{RNAi}/+;*Pnr-Gal4/UAS-Egr*; **F,** *dtak1*/+;*UAS-POSH*/+; *Pnr-Gal4*/+; **G**, *Ap-Gal4, UAS-dTAB2*/+; **H**, *Ap-Gal4, UAS-dTAB2/UAS-POSH* ^{RNAi}. In lines **B** and **G**, and *Pnr-Gal4/UAS-Egr* (not shown here), an irrelevant RNAi line *UAS-dTraf1-IR* (Xue et al., 2007) is also introduced to balance the Gal4 expression levels. In **C** and **D**, embryos are shown in lateral view, with the anterior to the left. Scale bar: 20 μm.

When *POSH* was expressed under the control of *Pnr-Gal4*, we found that the animals were embryonic lethal (Fig. 1C and Table 1). Since dTAK1 is a MAPKKK downstream of Egr and upstream of Hep in the Egr-JNK pathway and it can interact with POSH *in vitro* (Takatsu et al., 2000; Tsuda et al., 2005), we deleted one copy of *dTAK1* in *Pnr>POSH* flies and observed that it could partially suppress the over-expression-induced embryo lethality phenotype (Fig. 1I). The animals could survive to adulthood with merely thorax closure defects (Fig. 1F). These results suggest that dTAK1 is likely to play a role in the Egr signaling pathway downstream of POSH. Since *bsk* or *hep* null mutants are embryonic lethal, we tested whether reducing one copy of *bsk,* or the *hep* allele could rescue the lethality. Unexpectedly, none of these mutants were able to suppress the *Pnr>POSH* lethality phenotype (Fig. 1I). Co-overexpression of *bsk* or *hep* in the *Pnr>POSH* background could not suppress the dorsal closure defect either (data not shown).

dTAB2, an adaptor protein linking the Egr receptor Wengen to dTAK1 in the JNK pathway, also plays a role in *Drosophila* development (Geuking et al., 2005; Xue et al., 2007). Ectopic expression of *dTAB2* under the control of *Ap-Gal4*, a Gal4 driver expressed in dorsal compartments at the larval stage, affects thorax development (Fig. 1G). Down-regulation of *POSH* expression by RNAi significantly suppressed the scutellum defects of *Ap>dTAB2* (100% suppression, Fig. 1H), implying that POSH may act as the downstream component of dTAB2 in the TNF-JNK signaling pathway.

It has been shown that ectopic expression of *Egr* under the control of *GMR-Gal4* causes excessive cell death in the eyes leading to a small-eye phenotype (Igaki et al., 2002) (Fig. 2D), and over-expression of *POSH* in the eye with *GMR-Gal4* also results in a rough eye phenotype (Seong et al., 2001a) (Fig. 2C). Based on the above findings that POSH may play a role in Egr-JNK signaling during development, we speculated that POSH may be involved in Egr-JNK signaling-induced cell death. To test this hypothesis, we knocked down the expression of *POSH* by RNAi and found that the small-eye phenotype induced by *GMR>Egr* was significantly rescued (87%, Fig. 2E). Introducing of a null mutant $posh^{p327}$ we generated (described hereafter) heterozygously could also rescue the *GMR>Egr* phenotype by 37%. Again, this result suggests that POSH acts downstream of Egr. It is interesting to notice that over-expression of *POSH* could also suppress the small-eye phenotype of *GMR>Egr*, although a rough eye phenotype similar to that caused by *POSH* over-expression still existed (Fig. 2, C and F). Therefore, our results indicate that POSH plays an important role in the Egr-JNK pathway not only during *Drosophila* embryonic and thoracic development, but also in modulating Egr-JNK signaling-induced cell death in the eye.

Table 1
Embryonic lethality and cuticle defects in *posh* loss-of-function and over-expression mutants.

Genotype	N	Phenotypes (%)				
		Hatch to the 1st instar lavae	Fail of hatching	Dorsal hole and small anterior hole	Head involution defect	Germband retraction defect
k15815	171	0	75.5	11.1	5.8	7.6
p92	127	–*	70	4.9	9.4	15.7
p327	148	–	87.2	4.7	2	6.1
k15815/p92	80	12.5	62.4	6.3	8.8	10
k15815/p327	93	24.5	64.5	5.6	3.2	2.2
Df/p92	105	61.9	17.1	10.5	3.8	6.7
Df/p327	130	63.8	22.5	6.9	3	3.8
Df/k15815	114	8.8	58.8	20.2	5.2	7
Act>POSH	55	12.7	–	72	–	15.3
Pnr>POSH	110	–	–	81.8	–	18.2

* The number of such phenotype was not included in the statistical analysis.

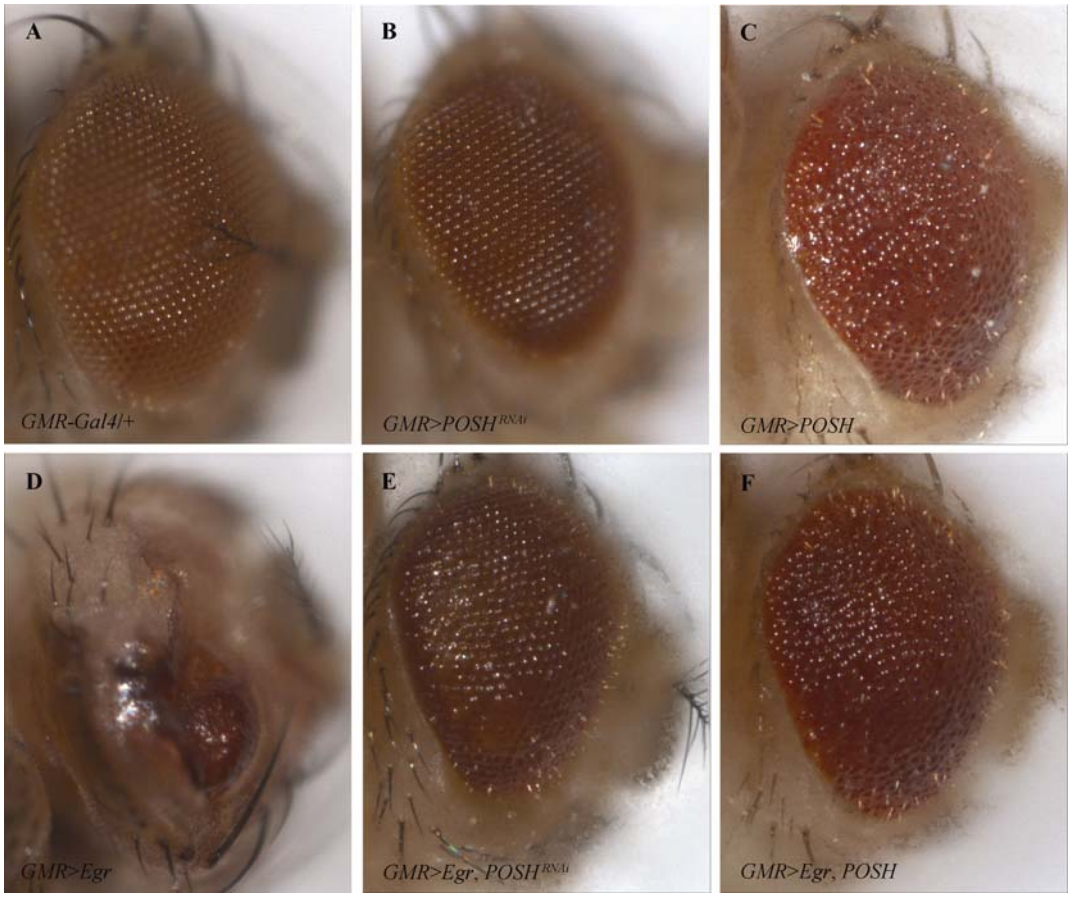

Fig. 2. POSH is required for *GMR>Egr*-induced cell death in the eye. **A:** wild-type eye. **B:** knockdown of *POSH* by RNAi under the control of *GMR-Gal4*. *GMR>POSH*^{RNAi} generates normal eyes of a slightly reduced size. **C:** over-expression of *POSH* in adult eyes. *GMR-Gal4* induced *UAS-POSH* over-expression resulting in rough eyes of a slightly larger size. **D:** ectopic expression of *Eiger* led to a small-eye phenotype. **E:** knockdown of *POSH* expression suppressed the small-eye phenotype induced by ectopic expression of *Eiger*. **F:** over-expression of *POSH* suppressed the small-eye phenotype induced by *Eiger* expression but the rough eye phenotype was not affected. Genotypes: **A**, *GMR-Gal4/+*; **B**, *GMR-Gal4/UAS-POSH*^{RNAi}; **C,** *GMR-Gal4/UAS-POSH*; **D**, *GMR-Gal4/+;UAS-Egr/+*; **E**, *GMR-Gal4/UAS-POSH*^{RNAi}*;UAS-Egr/+*; **F**, *GMR-Gal4/UAS-POSH;UAS-Egr/+*. In **A**–**D**, *UAS-dTraf1-IR* (Xue et al., 2007) is introduced as a control to balance the Gal4 expression levels.

In order to further confirm the role of POSH in the Egr-JNK pathway, we adopted *posh*^{74}, reported to be a null mutant bearing a deletion of the N-terminal 1,282 bases of *POSH* (Tsuda et al., 2005), and tried to rescue the ectopic Egr expression-induced phenotypes. However, *posh*^{74} could suppress neither the pupal lethality phenotype of *Pnr>Egr* nor the small-eye phenotype of *GMR>Egr*. We then did RT-PCR analysis and found that the transcript of the C-terminal part of *POSH* still existed in *posh*^{74} (data not shown and personal communications). This suggests that *posh*^{74} is not a complete null mutant.

Expression of POSH during Drosophila embryogenesis

Since the above results suggest that POSH plays a role during *Drosophila* development, we analyzed the expression pattern of POSH during embryogenesis by immunostaining. In accordance with the expression pattern determined by *in situ* hybridization (FlyExpress), POSH signal was distributed ubiquitously in the entire cytoplasm at early embryonic stages (Fig. 3A). As development proceeded, POSH was expressed mainly in the dorsal and ventral mesoderm at stages 9–10 during germ band retraction (Fig. 3B). At stages 13–14 the POSH signal was high in epidermal cells and could also be detected in amnioserosa cells (Fig. 3C). In animals with POSH expressed ectopically under the control of *Pnr-Gal4*, the expression pattern was similar albeit stronger than in control flies (Fig. 3D). During gastrulation, stronger signals were detected at epidermal cell borders (Fig. 3E), implying that POSH may

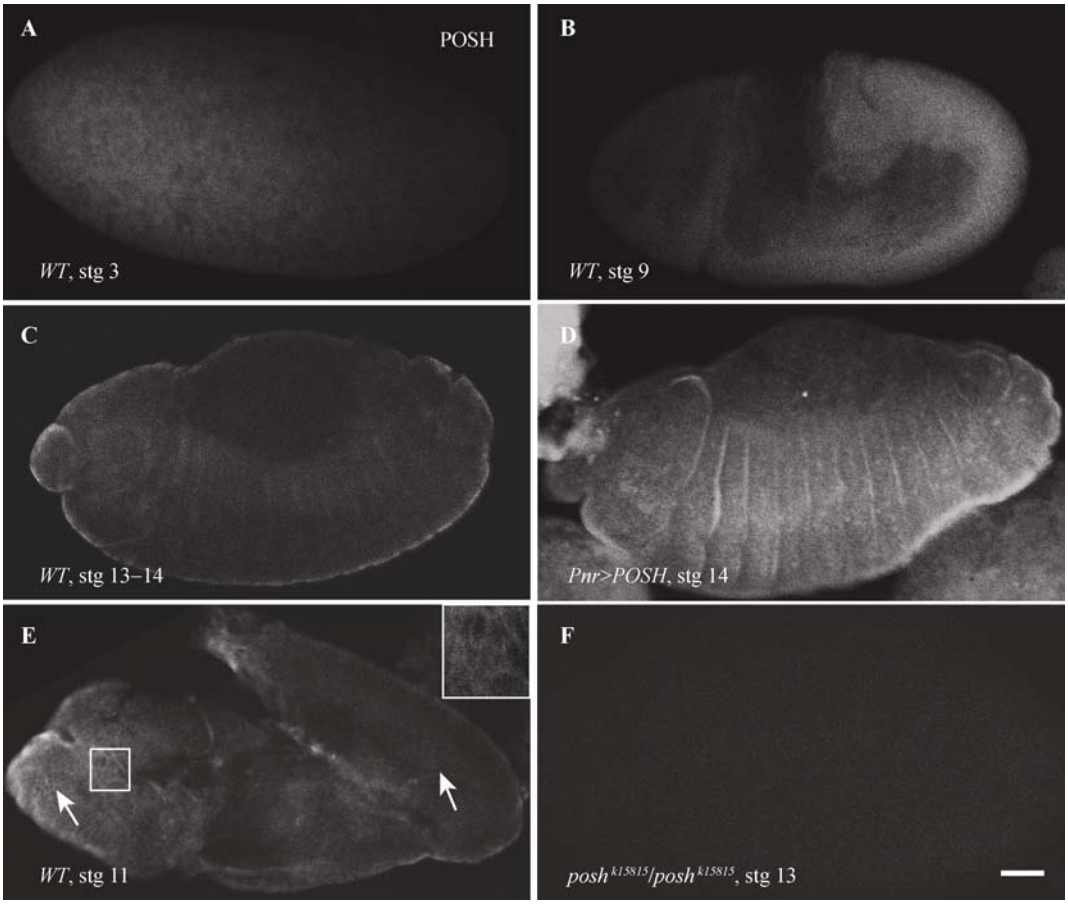

Fig. 3. Expression of POSH during embryogenesis. Embryos at different stages were immunostained with anti-POSH antibody. **A:** POSH is ubiquitously expressed at the pregastrulation stage. **B:** POSH is expressed mainly in the ectoderm during the gastrulation stage. **C:** expression of POSH in the epidermis during dorsal closure (DC). **D**: expression of POSH in *Pnr>POSH* during DC at stage 14. **E:** signals at the cell border during gastrulation (arrow, and in the white rectangle, upper right corner). **F:** negative POSH signal in the $posh^{k15815}$ homozygous mutant, at stage 13. Genotype: **A**–**C**, and **E**, yw; **D**, *UAS-POSH/+;Pnr-Gal4/+*; **F**, $posh^{k15815}/posh^{k15815}$. Embryos are shown in lateral view, with the anterior to the left. Images shown here are confocal sections. Scale bar: 50 μm.

be involved in cell-cell communication or cytoskeleton structure maintenance during embryo development.

Mutations in POSH result in abnormal embryonic morphogenesis

Based on the expression pattern of POSH during embryogenesis, and that knocking down *POSH* expression in zebrafish and mouse affects head development (Kim et al., 2005; manuscript in preparation, respectively), we postulated that POSH may play a role during early development. However, the $posh^{74}$ mutant has been reported to survive to adulthood (Tsuda et al., 2005), contrary to our prediction.

To clarify the exact role of POSH in development, we generated *posh* loss-of-function mutants through imprecise P-element excision of $posh^{EP1206}$, in which the P-element was inserted –270 bp upstream of the *POSH* start codon. The genome sequence coordinates for 8-bp insertion sites of P element EP1206 is GCCACGAG. Two null mutant strains $posh^{p92}$ and $posh^{p327}$, were confirmed by Western blotting and PCR sequencing (Fig. 4B and data not shown). In both mutants, small regions containing the start codon sequence were deleted (Fig. 4A). The flanking sequence of deleted sequence in $posh^{p92}$ and $posh^{p327}$ were: 5′-GCCAC GAG-3′ & 3′-TTCATCAT-5′ and 5′-GCCACGAG-3′ & 3′-ACCTAAAC-5′, respectively. Another lethal line $posh^{k15815}$, with a P-element inserted in the third exon (insertion site of P element k15815 is ATCTCAAG), did not express the C-terminal part of POSH either, as confirmed by Western blotting (Fig. 4B). All these alleles were lethal and showed various embryonic developmental defects (Fig. 5 and Table 1).

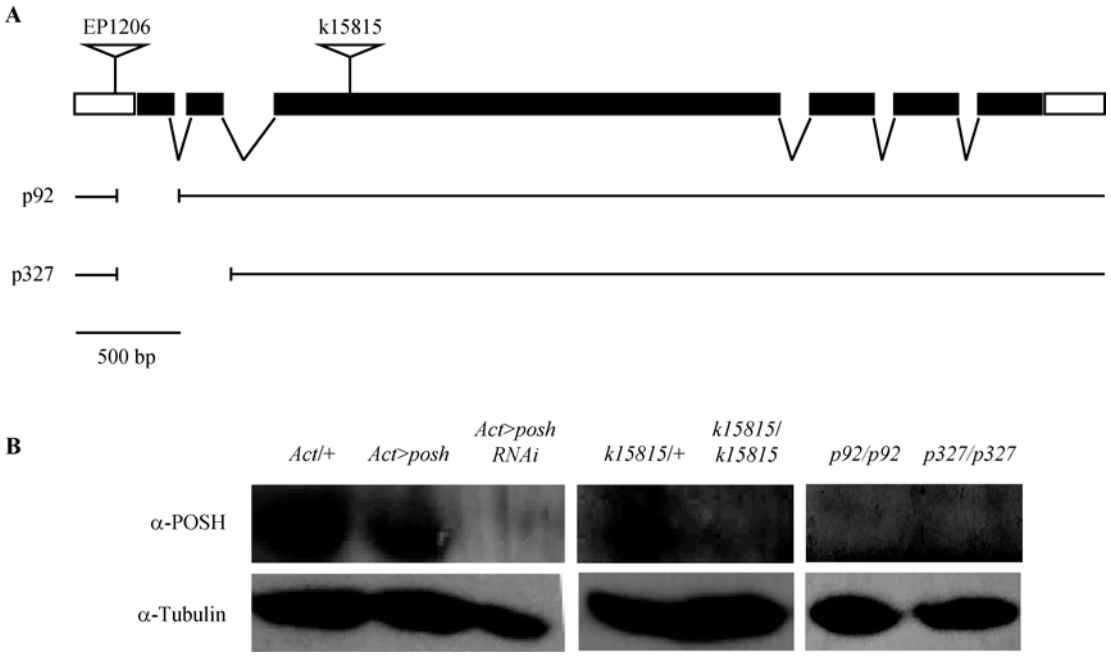

Fig. 4. Schematic representation of *POSH* and its mutations, and the expression of POSH protein in mutants. **A:** genomic organization of *POSH*. In $posh^{EP1206}$, a P-element is inserted upstream of *POSH*. In $posh^{k15815}$, a P-element is inserted in the third exon. The deletions $posh^{p92}$ and $posh^{p327}$ are depicted. Scale bar: 500 bp. **B:** expression of *POSH* in control, over-expression, and RNAi knockdown flies and in mutant alleles $posh^{k15815}$, $posh^{p92}$ and $posh^{p327}$ was analyzed with anti-POSH serum. Samples used in lane 1–3 were from adults and those in lane 4–7 were from stage 13–17 embryos.

Since JNK activity is required for dorsal closure during development (Riesgo-Escovar et al., 1996; Sluss et al., 1996; Geuking et al., 2005) and POSH has been shown as a required scaffold in regulating JNK activity (Tapon et al., 1998; Xu et al., 2003), we examined first whether these null alleles had dorsal closure defects. About 4%–12% of the mutants showed a dorsal hole in the epidermis (Table 1). Close inspection of mutant embryos stained for the cell border marker phospho-Tyrosine revealed the disruption of epidermal dorsal closure (Fig. 6B). In addition to the dorsal closure defect, germ band retraction, head involution and tracheal formation failures were also noticed in these *posh* null alleles (Fig. 5, Table 1). The null mutants, $posh^{p92}$, $posh^{k15815}$ and the genomic deficiency mutant *Df(2R)BSC45* could not complement each other (Table 1), and all trans-heterozygous alleles had similar phenotypes as the null mutants. Therefore, we concluded that POSH is required during *Drosophila* embryogenesis.

We next examined the *POSH* over-expression phenotype during embryogenesis. Over-expression of *POSH* in the whole body (driven by *Actin-Gal4* or *Tubulin-Gal4*) in *Drosophila* caused early stage lethality. Most of the flies died at the embryo stage and only a few could survive to 1st or 2nd stage larva. Over-expression of *POSH* under the control of other Gal4 drivers, for example, *Pannier-Gal4* which is expressed in dorsal tissues, amnioserosa and lateral epidermis, in embryos (Herranz and Morata, 2001), also resulted in embryonic lethality of animals with dorsal closure arrest (81.8% penetrance; Table 1, Fig. 1C, and Fig. 6B). In addition to the major dorsal closure defects, the embryos also had defects in germ band retraction (Table 1). Over-expression of *POSH* under the control of *Armadillo-Gal4* and *Engrailed-Gal4* showed similar phenotypes as *Pannier-Gal4* (data not shown).

Based on our observations, over-expression of *POSH* had a similar but more severe phenotype than the null mutants. In addition, over-expression of *POSH* under the control of the above Gal4 drivers could not rescue lethality and morphological defects in $posh^{p92}$ or $posh^{k15815}$ mutants. Therefore, we speculate, and will discuss in detail later, that over-expression of *POSH* may play a dominant-negative, other than gain-of-function role in the JNK pathway during embryogenesis.

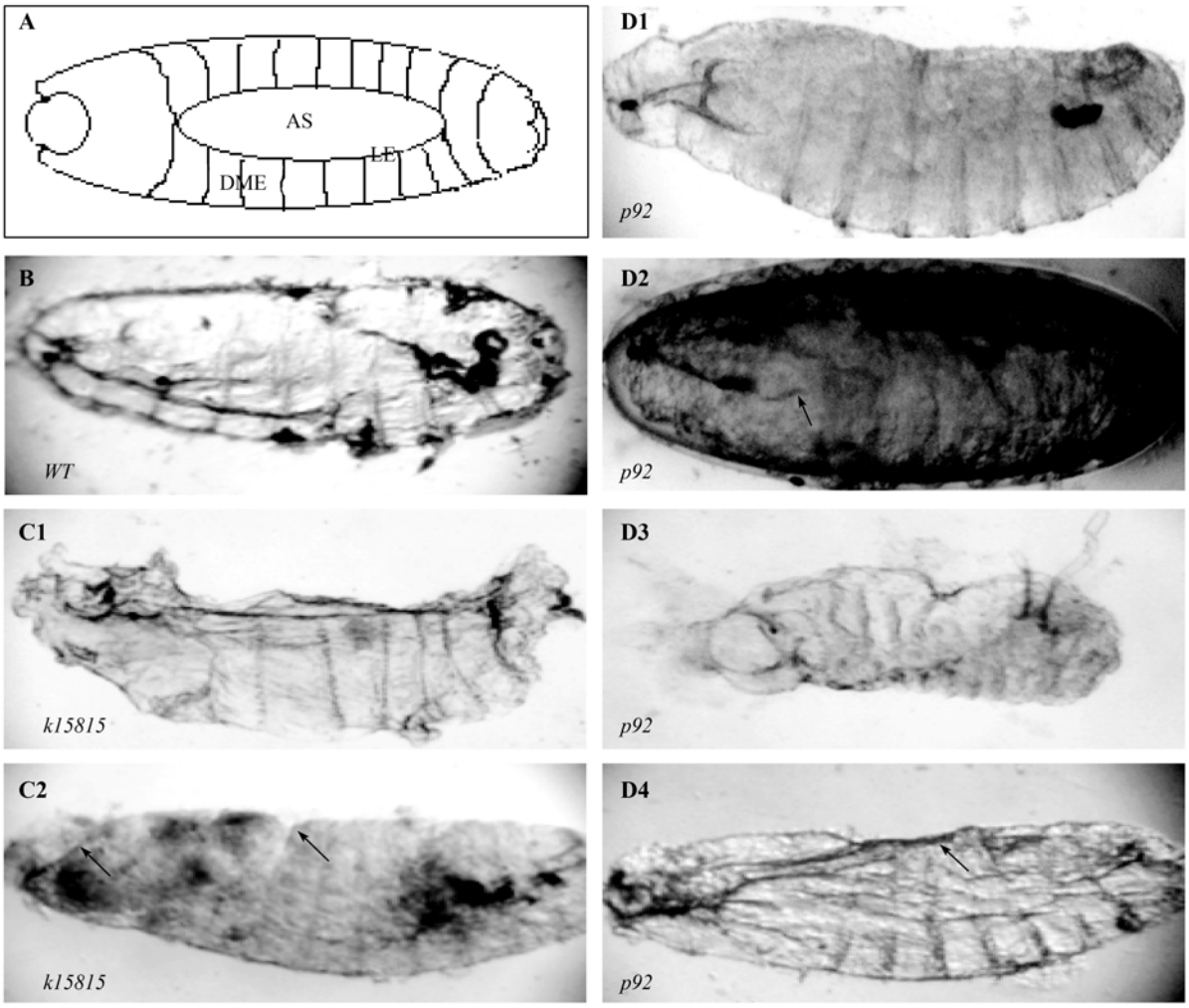

Fig. 5. Embryonic defects of *posh* mutants. **A:** a diagram shows the dorsal closure process. During DC, the dorsal-most epidermis (DME) stretches upwards to wrap around the amnioserosa (AS) and eventually sews it up like a zipper. The leading edge (LE) cells are the dorsal-most cells of the migrating epidermis. **B:** wild-type cuticle. **C:** *posh*^{k15815} homozygous mutant cuticle with dorsal closure defect (**C1**) or head involution defect (**C2**, left arrow). **D:** cuticle of *p92* homozygotes. Embryos can survive to the 1st instar larva (**D1**), embryo failed hatching, probably because of tracheal fill failure (**D2,** arrow), embryos with dorsal closure defects that have a puckered phenotype (**D3**) or dorsal closure defect (**D4**, arrow). Embryos are shown in a lateral view in **B**, **C2**, **D2** and **D3**. In **C1**, **D1** and **D4**, embryos are shown in a superior view. All embryos are displayed with the anterior to the left.

Requirement of POSH for epidermal cell migration and cell-shape change during dorsal closure

Due to its specific expression in the epidermis during embryogenesis, and the fact that the dorsal open phenotype displayed in *posh* mutants is very similar to the JNK pathway component mutants such as *hep* and *bsk* (Riesgo-Escovar et al., 1996; Sluss et al., 1996; Glise and Noselli, 1997), we focused on dorsal closure as a read-out to study the *in vivo* function of POSH in *Drosophila*.

During dorsal closure, epidermal cells undergo dramatic changes. Dorsal-most epidermal (DME) cells and epidermal cells elongate along the dorso-ventral axis to drive the epithelial sheets upwards dorsal-ventrally and then the epithelial sheets zip together along the dorsal midline, pushing amnioserosa beneath the epidermis (Lecuit and Lenne, 2007). To characterize the morphology defects of the *posh* mutant, we adopted a cell border marker, phospho-tyrosine (P-Tyr), to label the cell membrane of the dorsal-most epidermis cells, the actin-nucleating centers (ANCs) formed between neighboring DME cells and the amnioserosa (AS) cell boundaries (Kaltschmidt et al.,

2002). During dorsal closure in wild-type embryos, the actomyosin contractile cable formed, and the leading edge (LE) cells elongated dorsal-ventrally towards the mid-line and eventually merged (Fig. 6, A and C, yellow arrow). P-Tyr signal was high in LE, particularly at the contact point between adjacent LE cells (Fig. 6C, white arrow). As zipping proceeded, some of the AS cells became smaller due to squeezing each other, as shown by the staining of cell boundaries (Fig. 6C, double arrow head).

In many *posh* mutants, cell shape, leading edge, and amnioserosa cell changes showed abnormality during the cell sheet movement process (Fig. 6, E and G). In $posh^{k15815}$ and $posh^{p92}$ mutants that could not finish dorsal closure, the cell shape change and cell migration seemed to be roughly normal in some embryos (Fig. 6E, yellow arrow) but the epidermal sheets could not zip together (data not shown). In addition, DME cells and the leading edge showed reduced P-Tyr signals in these embryos (Fig. 6E, white arrow).

The phenotype in *POSH* over-expression flies was more severe. In *Pnr>POSH* embryos, we found two different abnormal cellular defects associated with dorsal closure arrest: the failure of DME cell elongation and unsuccessful AS contraction. As shown in Fig. 6G, instead of elongating, the DME cells maintained their scalloped shape (Fig. 6G, yellow arrow). P-Tyr staining was much weaker compared with the wild-type at the leading edge (Fig. 6G, white arrow), implying the failure of epidermal cell migration. The AS cells were loosely packed, instead of pressing against each other and shrinking. In addition, these cells showed apparently weaker and discontinuous P-Tyr signals along the cell boundaries (Fig. 6G, double arrow head). It is likely that the mutants lacked the force to drive the epidermis through the closing process. Based on these observations, we infer that POSH plays an important role in cell migration and cell-morphology change during dorsal closure.

POSH-dependent induction of F-actin accumulation and adherens junction maintenance during dorsal closure

During embryogenesis, specific ectodermal cells undergo coordinated shape changes to accommodate complex morphogenetic events such as invagination of neural precursors, extension and retraction of the germ band, dorsal closure and head involution. The dramatic cell shape changes and migration of the dorsal closure process is associated with the re-distribution of cytoskeletal proteins (Kaltschmidt et al., 2002). The observation of P-Tyr staining abnormalities in DME cells and LE of both *posh* loss-of-function and *POSH* over-expression mutants implies the existence of cytoskeletal abnormalities. We therefore examined the F-actin distribution during dorsal closure in *posh* mutants.

In wild-type animals, F-actin signals accumulated at the leading edge (Fig. 6D, white arrow), and active filopodiation could be detected at AS cell boundaries (Fig. 6D, yellow arrow). Among the mutants of $posh^{k15815}$ that could not finish dorsal closure, significant reduction of F-actin signals was observed in LE and at AS boundaries (Fig. 6F, at LE, white arrow; at AS boundary, yellow arrow). In *POSH* over-expression mutants under the control of *Pnr-Gal4*, F-actin signals were also reduced significantly and no clear phalloidin marked LE or epidermis structure could be observed (Fig. 6H, white and yellow arrows, respectively). Taken together, these results suggest that POSH is important for F-actin distribution and re-distribution during dorsal closure.

The shape and movement of contacting cells depends on cell-cell adhesion, contributed mainly by their dynamic interactions with cortical actin and actin organization (Lecuit and Lenne, 2007). During dorsal closure, both cytoskeleton structure and cell adhesion changes dramatically. JNK has been shown to play a role in cell-cell adhesion by forming a complex with E-cadherin/β-catenin and phosphorylating β-catenin to promote the formation of adherens junctions (Lee et al., 2009). We therefore investigated whether E-cadherin is involved in the dorsal closure defect in *posh* mutants.

An apparent decrease in the expression level of E-cadherin (E-cad) was observed in the *Actin-Gal4>POSH* cuticle. More apparent was the disruption of the normal distribution of E-cad along the AS cell boundaries. Instead of an evenly-distributed pattern of E-cad at adherens junctions (AJs), small particles tended to accumulate at cell-cell boundaries (Fig. 7B, arrow, and the upright rectangle), indicating the disruption of adherens junctions when *POSH* is over-expressed. Another adhesions junction marker, Discs Large (Dlg) which is localized bellow AJs also showed abnormal patterns in $posh^{k15815}$ null mutants. We found that the expression level of Dlg was also significantly decreased in DME (Fig. 7D, arrow head) and AS cells (Fig. 7D, arrow). These observations suggest that aberrant cell adhesion is likely to be responsible for the dorsal closure failure in *posh* mutants.

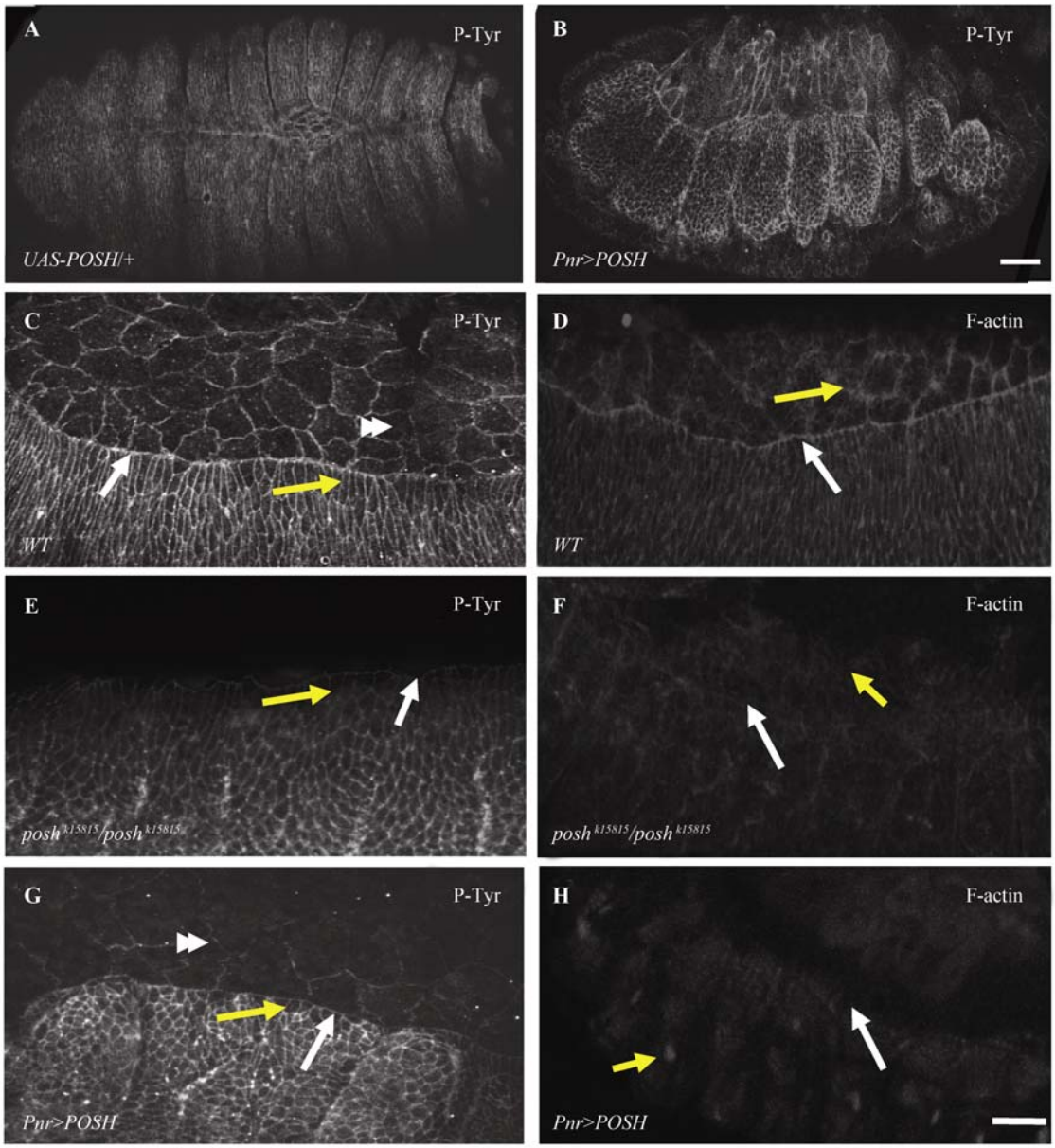

Fig. 6. F-actin is involved in POSH-dependant cell shape change and cell migration during DC. **A** and **B:** ectopic expression of *POSH* in the dorsal-most tissues results in dorsal closure failure. Stage 15 embryos were immunostained with phospho-Tyrosine (P-Tyr) antibody. **A:** wild-type control, LE marked by P-Tyr zipped up in the middle of the dorsal side of the embryo. **B:** *Pnr>POSH* embryo with defects in dorsal closure showing the failure of DME cell migration and unsuccessful AS cell contraction. **C, E** and **G:** POSH plays a role in cellular morphology changes during dorsal closure. In wild-type embryos (**C**), during stage 14–15, LE is shown by strong P-Tyr staining (white arrow), elongated DME cells (yellow arrow), and shrinking AS cells (double arrow head). In loss-of-function mutants (**E**), although some DME cells elongate normally (yellow arrow), the P-Tyr signals were significantly reduced in LE and DME cells (white arrow). **G:** *Pnr>POSH* mutant arrested at the beginning of the dorsal closure stage, displaying weak LE P-Tyr staining (white arrow), failed cell elongation (yellow arrow) and discontinuities at the AS cell boundary (double arrow head). **D, F** and **H:** F-actin structure is abnormal in *posh* mutants during DC. F-actin is labeled by phalloidin staining. In the wild-type (**D**), the F-actin signal is strong in LE and enlongated DME cells, indicating the activated state of DME cell elongation (white arrow). Filopodia can be detected at the AS cell boundaries (yellow arrow). In loss-of-function mutants (**F**), F-actin is much weaker and its structure is disorganized throughout the organism especially in epidermal tissues and AS cells (white arrow and yellow arrow). In over-expression animals (**H**), phalloidin staining was diffuse except at ANCs (yellow arrow), the LE was undetectable (white arrow). Genotypes: **A**, *UAS-POSH/+*; **B**, *UAS-POSH/+;Pnr-Gal4/+*; **C** and **D**, WT; **E** and **F**, $posh^{k15815}/posh^{k15815}$; **G** and **H**, *UAS-POSH/+;Pnr-Gal4/+*. Embryos are shown in a superior view in (**A**) and in a lateral view in **B**–**H**, with the anterior to the left. Scale bar, 50 μm in **A** and **B**, 10 μm in **C**–**H**.

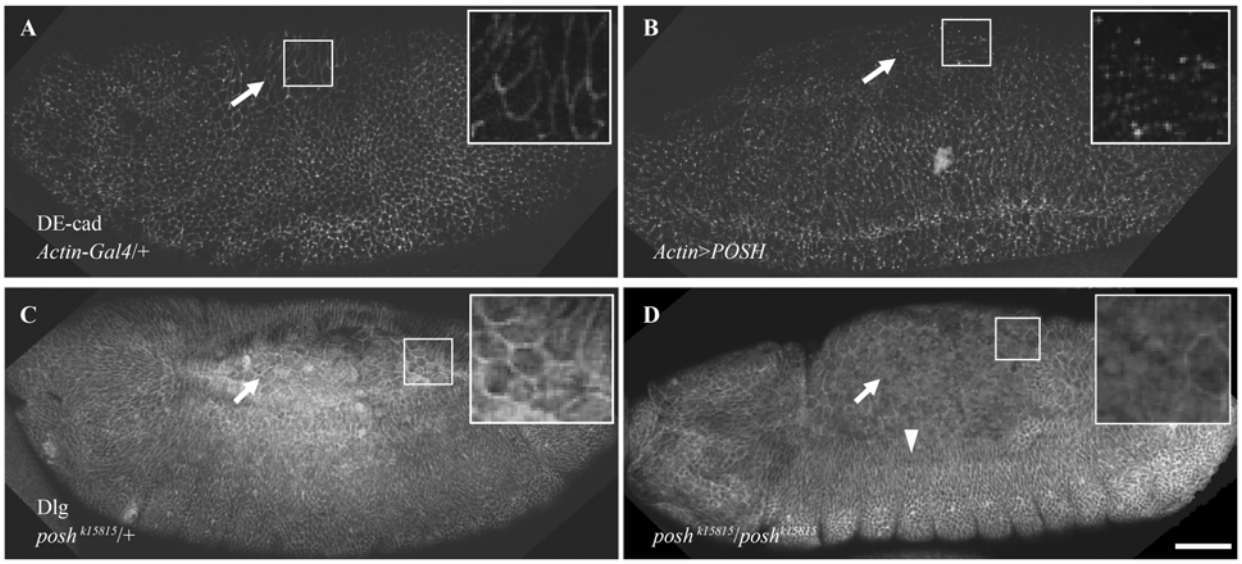

Fig. 7. The adherens junction is affected in *posh* mutants during DC. Compared with WT (**A**), E-cadherin expression level is lower in the *Actin-Gal4>UAS-POSH* cuticle (**B**), and the normal distribution of E-cad along the AS cell boundaries is disrupted (enlarged in the white rectangle, upper right corner). Compared to the heterozygous mutant (**C**), in loss-of-function mutants (**D**), Dlg staining signal decreased significantly in DME and AS cells (arrow) and the pattern (enlarged in the white rectangle, upper right corner) is also abnormal. Genotypes: **A**, *Actin-Gal4*/+; **B**, *UAS-POSH*/+;*Actin-Gal4*/+; **C**, $posh^{k15815}$/+; **D**, $posh^{k15815}/posh^{k15815}$. All embryos are shown in a lateral view, with the anterior end to the left. Scale bar, 50 μm.

Discussion

The importance of TNF signaling in mammalian systems is well recognized. Conservation of the components of the TNF-signal pathway has been confirmed in *Drosophila* (Igaki et al., 2002; Moreno et al., 2002; Geuking et al., 2005). However, whether POSH plays a role in TNF signaling, and its relationship with other components in this pathway, is still not clear. Although POSH has been suggested to interact with dTAK1 *in vitro* and play a role in the immune response in *Drosophila* (Tsuda et al., 2005), we found that the mutant used in that study was not a complete null mutant. It could not suppress the pupal lethality phenotype of *Pnr>Egr* and the small-eye phenotype of *GMR>Egr* in our study. Nevertheless, it is still reasonable to conclude from that study (Tsuda et al., 2005) that POSH, especially its N-terminal part, is essential for the immune response in *Drosophila*.

In this study, we investigated the role of POSH in the TNF-JNK signaling pathway in *Drosophila* and demonstrated that knockdown of *POSH* by RNAi suppressed the pupal stage lethality induced by *Pnr>Egr*, rescued the small-eye phenotype caused by *GMR>Egr* and significantly suppressed the scutellum defects of *Ap>dTAB2*. In addition, reduced expression of dTAK1 suppressed embryo lethality in *Pnr>POSH* flies. Together with previous reports that dTAK1, dTAB2 and JNK are involved in the apoptotic effect of Eiger (Igaki et al., 2002; Moreno et al., 2002) and that *POSH* over-expression induces JNK signaling through *bsk* (JNK) and *hep* (JNKK) (Seong et al., 2001a), we propose that POSH plays a role in the TNF/Eiger-JNK signaling pathway and that POSH is likely to act downstream of TNF/Eiger and dTAB2 but upstream of dTAK1.

We were surprised to find that over-expression of *POSH* could also suppress the small-eye phenotype of *GMR>Egr*. In addition, it was shown recently that the dominant-negative form of *dTAK1*, but not the reduction of JNK pathway signaling, suppresses the *POSH* over-expression induced rough eye phenotype (Lennox and Stronach, 2010). These results raise the possibility that POSH may play roles in alternative signaling pathways that suppress each other. POSH has been shown to be able to activate both the JNK and NF-κB pathways (Tapon et al., 1998; Xu et al., 2003, 2005). Activation of the NF-κB pathway can induce the expression of genes to promote cell proliferation. It can also suppress cell death through inhibition of the JNK pathway (De Smaele et al., 2001; Tang et al.,

2001). Therefore, the rough eye phenotype caused by over-expression of *POSH* is likely due to the activation of NF-κB pathway-induced cell proliferation. Over-expression of *POSH* may also suppress cell death incurred by *GMR>Egr* through the activation of the NF-κB pathway in the eye. This would also explain why over-expression of *POSH*, but not down-regulation of *bsk*, suppresses the small-eye phenotype of *GMR>Egr*.

The specific expression of POSH in the epidermis during embryogenesis may account for the failure of dorsal closure in *posh* null mutants. The phenotype is very similar to other mutants of the JNK signaling pathway such as *hep*, *bsk* and *slpr* (Glise et al., 1995; Riesgo-Escovar et al., 1996; Sluss et al., 1996; Stronach and Perrimon, 2002). In mammalian cells, POSH can interact with their mammalian homolog and regulate JNK signaling (Xu et al., 2003). It is therefore reasonable to postulate that POSH plays an essential role during embryogenesis through regulation of the JNK pathway.

It is interesting to note that over-expression of *POSH* caused similar dorsal closure defects as the *posh* null mutants and had even higher penetrance. In addition, over-expression of *POSH* could not rescue lethality and morphological defects in $posh^{p92}$ or $posh^{k15815}$ mutants. This is reminiscent of a study on another JNK pathway scaffold protein, JIP-1 (Yasuda et al., 1999). JIP-1 was originally identified as a JNK interacting protein that could inhibit the JNK pathway. Later, it was realized that over-expression of JIP-1 plays a dominant-negative role in the JNK signaling pathway. Based on our observations, we might assume that over-expression of *POSH*, similar to JIP-1 over-expression, plays a dominant-negative role in the JNK pathway during embryogenesis. In support of our speculation, expression of dominant-negative *dTAK1* under the control of *Pnr-Gal4* results in embryo lethality with a dorsal hole (Mihaly et al., 2001), which is very similar to the phenotype of *Pnr>POSH*.

During the preparation of this manuscript, Lennox and Stronach (2010) reported the same dorsal open phenotype in embryos with ectopically expressed *POSH*. They proposed that the defect is due to *Drosophila* caspase-9 homolog-dependent apoptosis of the amnioserosa. However, our findings indicate that, during dorsal closure, both ectopic expression and loss-of-function *posh* mutants have problems with DME cell elongation, AS cell contraction and lateral dorsal epithelial upward movement. In addition to defects in cell shape change, P-Tyr staining levels are decreased at the leading edge, the DME cells and the AS cell boundaries. These findings indicate that POSH plays an important role in cell migration and in cell morphology change during dorsal closure. The high penetrance and more severe dorsal closure defect caused by *POSH* over-expression is likely due to its dominant-negative role in the JNK pathway in addition to induction of cell death. And the findings that JNK and POSH could affect neuron migration in mouse embryo brain provided evidences that POSH has an important role in the process of cell migration (Sun et al., 2007; data unpublished).

JNK signaling has been shown to be crucial for F-actin-based filopodia and lamellipodia formation in the epithelial cells of the leading edge (Kaltschmidt et al., 2002). JNK activation is involved in actin condensation in the leading edge and the lateral dorsal epidermis, the movement of epithelial sheets and dorsal closure in *Drosophila* (Jacinto et al., 2002; Martin and Wood, 2002). Similar to mutants of the JNK pathway, *posh* mutants showed failed organization of the F-actin cable, lower levels of the F-actin signal at the leading edge and epidermis, and reduction of F-actin-rich filopodia at the leading edge. These results indicate that POSH may regulate cytoskeleton dynamics and formation of filopodia during dorsal closure through JNK signaling.

E-cadherin-mediated adhesion is stabilized by the adaptor proteins, α-catenin and β-catenin, and actin-filaments (Jamora and Fuchs, 2002; Bershadsky, 2004; Gates and Peifer, 2005). On the other hand, F-actin cable formation also depends on adherens junctions (Takahashi et al., 2005). Through the regulation of the E-cadherin/β-catenin complex, JNK plays an important role in controlling cell-cell adhesion (Lee et al., 2009). We have shown that *posh* mutants display abnormal patterns and decreased expression levels of E-cad and Dlg during dorsal closure. This suggests that POSH is required for cell morphology maintenance and cell migration through the control of adhesions junction formation. The regulatory function of POSH is probably performed *via* the JNK pathway, however, biochemical evidence is still needed to support this conclusion.

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