Research Article

Loss of flfl Triggers JNK-Dependent Cell Death in Drosophila

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The JNK pathway is evolutionary conserved from Drosophila to mammal [10]. As its genome has low redundancy, Drosophila has been used as an excellent genetic model to study tumor necrosis factor- (TNF-) induced cell death in development. In Drosophila, the TNF ortholog Eiger (Egr) triggers cell death through its receptor Grindelwald (Grnd) [11], the E2 ubiquitin conjugating enzyme complex Bendless/dUev1a [12, 13], the E3 ubiquitin ligase dTRAF2 [14], the TAK1-associated binding protein 2 Tab2 [15], and the dTAK1-Hep-Bsk (Drosophila homologs of JNKK-JNKK-JNK) kinase cascade [16, 17]. In developing eyes, ectopically expressing Egr by GMR-Gal4 (GMR > Egr hereafter) induces JNK-dependent cell death and produces small eyes in adult [16, 17].

To identify additional factors that regulate Egr-triggered JNK-mediated cell death, we performed a genetic screen for dominant modifiers of the GMR > Egr small eye phenotype. From the screen, we found that expression of flfl suppresses Egr-triggered cell death. On the other hand, knocking down flfl induced JNK activation and JNK pathway-dependent cell death, suggesting a physiological function of flfl in animal development. To our knowledge, this is the first report that flfl negatively regulates TNF-JNK signaling-induced cell death in vivo.

2. Materials and Methods

2.1. Drosophila Strains. All stocks were raised on standard Drosophila media, and crosses were performed at 25°C. UAS-flfl-IR (V103793) was obtained from Vienna Drosophila Research Center, UAS-flfl-IR (31690), flflEV03585, UAS-GFP-IR, and ap-Gal4 were obtained from Bloomington Stock.
### 3. Results and Discussion

#### 3.1. flf Suppresses Egr-Induced Cell Death in Eye Development

As previous study showed, ectopic expression of Egr under the control of GMR-Gal4 induced a small eye phenotype [17]. This phenotype is mostly suppressed by coexpressing a dominant negative allele of Bsk (Bsk^{DN}) encoding the Drosophila JNK ortholog [21], which indicates Egr-induced cell death is mainly mediated by JNK signaling [25]. To identify additional components of the Egr-JNK pathway or factors interacting with the pathway, we performed a genetic screen for dominant modifiers of the GMR > Egr small eye phenotype and identified Nopo, Ben, Wnd, and Wg signaling as essential regulator of Egr-JNK pathway induced cell death [21, 26].

From the screen, we also found that the GMR > Egr small eye phenotype (Figure 1(c)) was significantly suppressed by flf^{EY03585} (Figure 1(e)), a P-element inserted in the first intron of flf. This P-element carries the UAS sequence located about 1kb upstream of the coding region and is able to drive the expression of flf by the GMR-Gal4 driver. However, expression of flf by itself had no effect on the eye size (Figure 1(b)), compared to the GMR-Gal4 control (Figure 1(a)). As a negative control, coexpressing GFP did not suppress GMR > Egr-triggered small eye phenotype (Figure 1(d)). Thus, the data indicate that flf is able to suppress Egr-induced cell death in the eye.

#### 3.2. Loss of flf Enhances Egr-Induced Cell Death in Eye Development

As flf gain of function suppressed Egr-induced cell death, we wonder whether loss of flf could enhance Egr-triggered cell death. To this end, we knocked down flf in the eye by expressing flf RNAi with GMR-Gal4 and observed a rough eye phenotype (Figure 2(d)), compared to the control (Figure 2(a)). Consistent with previous reports, expression of a weaker UAS-Egr allele (UAS-Egr^{w}) driven by GMR-Gal4 resulted in a rough eye phenotype (Figure 2(b)). This phenotype is severely enhanced by knocking down flf as there was almost no eye tissue left (Figure 2(e)). As a negative control, expressing a RNAi sequence specifically targeting green fluorescent protein (GFP) has no effect on GMR > Egr^{w}-triggered rough eye phenotype (Figure 2(c)).
results show that flfl loss of function rigorously enhances Egr-triggered eye phenotype.

It was previously reported that ectopic Egr-induced eye phenotype is caused by cell death [16]. To examine cell death in vivo, we performed acridine orange (AO) staining that specifically labels dying cell. As reported previously [12], ectopic expression of a weak UAS-Egr transgene (UAS-Egr\(^w\)) driven by GMR-Gal4 induced mild cell death in eye discs posterior to the morphogenetic furrow (MF), as revealed by AO staining (Figure 2(g)). Egr-triggered cell death was rigorously enhanced by expressing flfl RNAi (Figure 2(j)) but remained unaffected by expressing GFP RNAi (Figure 2(h)). Consistent with its rough eye phenotype, knocking down flfl provoked weak cell death (Figure 2(i)). These data suggest that loss of flfl enhances Egr-induced cell death in eye development.

### 3.3. Loss of flfl Enhances JNK-Mediated Cell Death in Thorax Development

To investigate whether flfl suppresses JNK-mediated cell death in other tissues, we activated JNK signaling in the notum with pannier-Gal4 (pnrr-Gal4). Expression of Hep, the Drosophila homolog of JNK, driven by pnrr-Gal4 induced cell death and produced a small scutellum in adult fly (Figure 3(d)) [21]. Knocking down flfl by pnrr-Gal4 slightly decreased scutellum size (Figure 3(c)) and dramatically enhanced Hep-induced cell death by producing a no scutellum phenotype as well as a split thorax in adult flies (Figure 3(f)). As a negative control, expression of a GFP RNAi did not produce any effect on scutellum size (Figures 3(b) and 3(e)). Together, the results indicated that flfl negatively regulates JNK-mediated cell death in thorax development.

During Drosophila imaginal discs development, slow-proliferating cells are eliminated by a process called “cell competition” [27], which regulates tissue’s homeostasis and organs’ fitness and final cell number. JNK pathway was shown to play a crucial role in cell competition by eliciting cell death in “loser cells” [28, 29]. Since our data suggest that flfl impedes JNK-mediated cell death in a nontissue specific manner, flfl is likely a negative regulator of JNK-dependent cell competition and tissue homeostasis.

### 3.4. Loss of flfl Induces JNK Pathway Activation and Cell Death in Wing Development

To investigate the physiological functions of flfl in wing development, we specifically knocked down flfl in the posterior compartment of wing discs by en规则ed-Gal4 (en-Gal4) and checked cell death with AO staining. We found that loss of flfl triggered extensive cell death in the posterior compartment of wing discs (Figure 4(c)), compared with the en-Gal4 control (Figure 4(a)) and en > GFP-IR (Figure 4(b)). These results suggest that flfl is physiologically required for cell survival in Drosophila wing development.

To examine whether JNK signaling plays a role in loss of flfl induced cell death, we checked the expression of puc, a transcriptional target of JNK pathway [30]. puc\(^E69\) is a puc mutant allele with a LacZ bearing P-element inserted into the puc locus and serves as a puc-LacZ reporter [31] whose expression could be easily visualized by X-Gal staining. We found that knocking down flfl in the posterior compartment of wing discs resulted in upregulated puc-LacZ expression (Figure 4(f)), compared with the en-Gal4 control.
Figure 3: Loss of \textit{flfl} enhances JNK-mediated cell death in thorax. Light images of \textit{Drosophila} adult thoraxes are shown. Compared with the wild type (a) and \textit{pnr > GFP-IR} control (b), expression of Hep induced a small scutellum (d), which was dramatically enhanced by the expression of \textit{flfl} RNAi (f), while expression of \textit{flfl} RNAi slightly decreased scutellum size (c). Dashed rectangle indicates the scutellum. Genotypes: \textit{pnr-Gal4/+ (a); UAS-GFP-IR/+; pnr-Gal4/+ (b); UAS-flfl-IR/+; pnr-Gal4/+ (c); UAS-Hep/+; pnr-Gal4/+ (d); UAS-Hep/UAS-GFP-IR; pnr-Gal4/+ (e); UAS-Hep/UAS-flfl-IR; pnr-Gal4/+ (f).}

(\textit{Figure 4(d)}) and \textit{en > GFP-IR} (\textit{Figure 4(e)}), suggesting that loss of \textit{flfl} promotes JNK pathway activation.

The JNK pathway is evolutionarily conserved from fly to human. Compared with the compact \textit{Drosophila} genome, there are three homologs of \textit{flfl}, SMEK1, SMEK2, and SMEK3P, and dozens of Puc homologs named dual specificity phosphatase (DUSP) in human. Previous study has reported that JNK signaling is essential for cell migration and tumor invasion [32]. Based on the above data, we speculate that SMEK is downregulated and DUSP is upregulated in metastatic tumor. Consistent with the hypothesis, we found from the Oncomine database (https://www.oncomine.org/) that SMEK1 expression is indeed downregulated whereas DUSP1 is upregulated in invasive breast carcinoma stroma compared to normal tissue (\textit{Figures 4(g) and 4(h)}) [33]. These data imply that the role of \textit{flfl} in modulating JNK pathway is likely conserved by SMEK1 from \textit{Drosophila} to human.

Although our data mining and previous study found that JNK activity is elevated in several cancer cell lines, its role in tumor development is context-dependent [8]. JNK pathway was implicated as both pro- and anticancer signaling in cancer development for its regulation on cell proliferation and cell death, respectively [6]. In certain mouse models of cancer, JNK deficiency enhances tumor formation and metastasis [20, 34]. In \textit{Drosophila}, clones with ectopic oncogene \textit{Src} expression induce no-autonomous tumor growth [35], while \textit{Src} expression also induces cell death through JNK pathway [22]. Cells in \textit{Src} clone could escape from cell death if JNK pathway is blocked [35]. Intriguingly, another important oncogene Ras can also switch JNK pathway from anti- to protumor signaling [6]. Thus, upon the presence of different regulating factor(s), JNK pathway modulates cell death, tumor genesis, and progression in a cell context-dependent manner.

\subsection*{3.5. Loss of \textit{flfl} Induced Cell Death Is JNK Pathway-Dependent.}

Knocking down \textit{flfl} by \textit{GMR-Gal4} induced cell death in eye discs (\textit{Figure 2(i)}) and produced a rough eye phenotype in adults (\textit{Figure 2(d)}). These results were confirmed by another independent line of \textit{flfl} RNAi (\textit{Figures 5(b) and 5(b')}). To understand whether loss of \textit{flfl} induced cell death is JNK pathway dependent, we blocked JNK signaling by expressing a \textit{bsk} RNAi or a dominant negative allele of \textit{Bsk (Bsk^{DN})}. We found that loss of \textit{flfl} triggered rough eye phenotype (\textit{Figure 5(b)}) and increased cell death in eye discs (\textit{Figure 5(b')}) were significantly suppressed by compromised JNK activity (\textit{Figures 5(c)–5(e)}). As a control, \textit{GFP RNAi} and
Figure 4: Loss of $\textit{flfl}$ induces JNK pathway activation and cell death in wing development. *Drosophila* 3rd instar wing discs with AO ((a)–(c)) and X-Gal staining ((d)–(f)) are shown. Knocking down $\textit{flfl}$ in the posterior compartment of wing discs by en-Gal4 induced extensively cell death (c) and $\textit{puc}$-LacZ expression (f), while expressing a GFP RNAi failed to do so ((b) and (d)). en-Gal4 ((a) and (d)) served as controls. Dashed line indicates the anterior-posterior boundary of wing discs ((c) and (f)). Anterior boundary is to the left in all panels. Genotypes: en-Gal4/+ (a); en-Gal4/UAS-GFP-IR (b); en-Gal4/UAS-\textit{flfl}-IR (c); en-Gal4/+; $\textit{puc}^{69}$/+ (d); en-Gal4/+; $\textit{puc}^{69}$/UAS-GFP-IR (e); en-Gal4/+; $\textit{puc}^{69}$/UAS-\textit{flfl}-IR (f). SMEK1 (g) and DUSP1 (h) relative expression level in invasive breast carcinoma stroma compared to normal tissue in Finak Breast dataset are shown. Reporter: A:\textsubscript{24} P36961 and A:\textsubscript{23} P110712 are probes used in the study to detect SMEK1 and DUSP1, respectively. Breast stands for normal samples. The number in the parenthesis represents the total number of samples.
Figure 5: flf fl loss-of-function induced cell death was suppressed by compromised JNK activity. Light micrographs of Drosophila eyes ((a)–(d)) or acridine orange staining of eye discs from 3rd instar larvae ((a')–(d') and (f')–(h')) are shown. Compared with the control (a), knocking down flf fl induced cell death in eye discs (b') and a rough eye phenotype in adult (b), which were significantly suppressed by knocking down bsk ((c') and (c)) or coexpressing a dominant negative form of Bsk ((d') and (d)). Expressions of GFP-IR, bsk-IR, or BskDN were included as controls ((f)–(h')). (e) is the statistical analysis of acridine orange positive cells in the posterior part of eye discs from the indicated panels. Column shows mean + SEM and significance was tested by unpaired Student t-test; **P ≤ 0.01; ***P ≤ 0.001.

loss of Bsk signaling produced no evident phenotype in adult eyes (Figures 5(f)–5(h)). These results indicate that depletion of flf fl induced cell death is JNK pathway-dependent.

4. Conclusions

In this study we have identified flf fl as a negative regulator of TNF-trigger JNK-mediated cell death in Drosophila. While ectopic expression of flf fl impedes JNK signaling-induced cell death, loss of flf fl induces JNK pathway activation and cell death in Drosophila eye and wing discs and produced morphological defects in the adult eye. These data suggest an important physiological function of flf fl in maintaining tissue homeostasis in Drosophila organ development. flf fl’s ability to inhibit JNK signaling is likely retained by its human homolog SMEK1. Consistently, while activated JNK pathway promotes dermal fibroblasts cell migration in wound healing [36], ectopic expression of SMEK1 significantly decreased the migration ability of carcinoma cells [37]. In addition, we found from Oncomine database that SMEK1 is downregulated whereas JNK signaling target gene DUSP1 is upregulated in human invasive carcinoma [33].

Conflict of Interests

The authors declare no conflict of interests.
Acknowledgments

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References

[1] A.-C. Gingras, M. Caballero, M. Zarske et al., “A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity,” Molecular & Cellular Proteomics, vol. 4, no. 11, pp. 1725–1740, 2005.

[2] R. Sousa-Nunes, W. Chia, and W. G. Somers, “Protein Phosphatase 4 mediates localization of the Miranda complex during Drosophila neuroblast asymmetric divisions,” Genes and Development, vol. 23, no. 3, pp. 359–372, 2009.

[3] Z. Lipinszki, S. Lefevre, M. S. Savoian, M. R. Singleton, D. M. Glover, and M. R. Przewloka, “Centromeric binding and activity of protein phosphatase 4,” Nature Communications, vol. 6, article 5894, 2015.

[4] G. Zhou, K. A. Mihindukulasuriya, R. A. MacCorkle-Chosnek et al., “Protein phosphatase 4 is involved in tumor necrosis factor-α-induced activation of c-Jun N-terminal kinase,” The Journal of Biological Chemistry, vol. 277, no. 8, pp. 6391–6398, 2002.

[5] L. Chen, W. Dong, T. Zou et al., “Protein phosphatase 4 negatively regulates LPS caspase by inhibiting ubiquitination of TRAF6,” FEBS Letters, vol. 582, no. 19, pp. 2843–2849, 2008.

[6] M. Enomoto, D. Kizawa, S. Ohsawa, and T. Igaki, “JNK signaling is converted from anti- to pro-tumor pathway by Ras-mediated switch of Warts activity,” Developmental Biology, vol. 403, no. 2, pp. 162–171, 2015.

[7] S. Ohsawa, K. Sugimura, K. Takino, T. Xu, A. Miyawaki, and T. Igaki, “Elimination of oncogenic neighbors by JNK-mediated engulfment in Drosophila,” Developmental Cell, vol. 20, no. 3, pp. 315–328, 2011.

[8] C. R. Weston and R. J. Davis, “The JNK signal transduction pathway,” Current Opinion in Cell Biology, vol. 19, no. 2, pp. 142–149, 2007.

[9] J. Lyu, X. Yu, L. He et al., “The protein phosphatase activity of PTEN is essential for regulating neural stem cell differentiation,” Molecular Brain, vol. 8, no. 1, article 26, 2015.

[10] Y.-C. Su, J. E. Treisman, and E. Y. Skolnik, “The Drosophila Ste20-related kinase misshapen is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway,” Genes and Development, vol. 12, no. 15, pp. 2371–2380, 1998.

[11] D. R. Schwartz, S. L. R. Kardia, K. A. Shedden et al., “Gene expression in ovarian cancer reflects both morphology and biological behavior, distinguishing clear cell from other poor-prognosis ovarian carcinomas,” Cancer Research, vol. 62, no. 16, pp. 4722–4729, 2002.

[12] X. Ma, L. Yang, Y. Yang, M. Li, W. Li, and L. Xue, “dUevla modulates TNF-JNK mediated tumor progression and cell death in Drosophila,” Developmental Biology, vol. 380, no. 2, pp. 211–221, 2013.

[13] X. Ma, W. Li, H. Yu et al., “Bendless modulates JNK-mediated cell death and migration in Drosophila,” Cell Death and Differentiation, vol. 21, no. 3, pp. 407–415, 2014.

[14] L. Xue, T. Igaki, E. Kuranaga, H. Kanda, M. Miura, and T. Xu, “Tumor suppressor CYLD regulates JNK-induced cell death in Drosophila,” Developmental Cell, vol. 13, no. 3, pp. 446–454, 2007.

[15] P. Geuking, R. Narasimamurthy, and K. Basler, “A genetic screen targeting the tumor necrosis factor/eiger signaling pathway: identification of drosophila TAB2 as a functionally conserved component,” Genetics, vol. 171, no. 4, pp. 1683–1694, 2005.

[16] T. Igaki, H. Kanda, Y. Yamamoto-Goto et al., “Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway,” The EMBO Journal, vol. 21, no. 12, pp. 3009–3018, 2002.

[17] E. Moreno, M. Yan, and K. Basler, “Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily,” Current Biology, vol. 12, no. 14, pp. 1263–1268, 2002.

[18] M. Fanto, U. Weber, D. I. Strutt, and M. Mlodzik, “Nuclear signaling by Rac and Rho GTPases is required in the establishment of epithelial planar polarity in the Drosophila eye,” Current Biology, vol. 10, no. 16, pp. 979–988, 2000.

[19] M. Motwani, F. M. Sirotnak, Y. She, T. Commes, and G. K. Schwartz, “Drgl, a novel target for modulating sensitivity to CPT-11 in colon cancer cells,” Cancer Research, vol. 62, no. 14, pp. 3950–3955, 2002.

[20] Q.-B. She, N. Chen, A. M. Bode, R. A. Flavell, and Z. Dong, “Deficiency of c-Jun-NH2-terminal kinase-1 in mice enhances skin tumor development by 12-O-tetradecanoylphorbol-13-acetate,” Cancer Research, vol. 62, no. 5, pp. 1343–1348, 2002.

[21] S. Zhang, C. Chen, C. Wu, Y. Yang, W. Li, and L. Xue, “The canonical Wg signaling modules Bsk-mediated cell death in Drosophila,” Cell Death and Disease, vol. 6, no. 4, Article ID e1713, 2015.

[22] X. Ma, Y. Shao, H. Zheng, M. Li, W. Li, and L. Xue, “Src42A modulates tumor invasion and cell death via Ben/dUevla-mediated JNK activation in Drosophila,” Cell Death and Disease, vol. 4, no. 10, article e864, 2013.

[23] L. Xue and M. Noll, “Dual role of the Pax gene paired in accessory gland development of Drosophila,” Development, vol. 129, no. 2, pp. 339–346, 2002.

[24] M. J. Bertram, G. A. Akkerk, R. L. Ard, C. Gonzalez, and M. F. Wolfiner, “Cell type-specific gene expression in the Drosophila melanogaster male accessory gland,” Mechanisms of Development, vol. 38, no. 1, pp. 33–40, 1992.

[25] T. Igaki and M. Miura, “The Drosophila TNF ortholog Eiger: emerging physiological roles and evolution of the TNF system,” Seminars in Immunology, vol. 26, no. 3, pp. 267–274, 2014.

[26] X. Ma, J. Huang, L. Yang, Y. Yang, W. Li, and L. Xue, “NOPO modulates Egr-induced JNK-independent cell death in Drosophila,” Cell Research, vol. 22, no. 2, pp. 425–431, 2012.

[27] L. Baillon and K. Basler, “Reflections on cell competition,” Seminars in Cell & Developmental Biology, vol. 32, pp. 137–144, 2014.

[28] E. Moreno, K. Basler, and G. Morata, “Cells compete for deca-pentaplegic survival factor to prevent apoptosis in Drosophila wing development,” Nature, vol. 416, no. 6882, pp. 755–759, 2002.
[29] E. Moreno and K. Basler, “dMyc transforms cells into super-competitors,” Cell, vol. 117, no. 1, pp. 117–129, 2004.

[30] E. Martín-Blanco, A. Gampel, J. Ring et al., “Puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in Drosophila,” Genes & Development, vol. 12, no. 4, pp. 557–670, 1998.

[31] F. Agnès, M. Suzanne, and S. Noselli, “The Drosophila JNK pathway controls the morphogenesis of imaginal discs during metamorphosis,” Development, vol. 126, no. 23, pp. 5453–5462, 1999.

[32] C. Huang, K. Jacobson, and M. D. Schaller, “MAP kinases and cell migration,” Journal of Cell Science, vol. 117, part 20, pp. 4619–4628, 2004.

[33] G. Finak, N. Bertos, F. Pepin et al., “Stromal gene expression predicts clinical outcome in breast cancer,” Nature Medicine, vol. 14, no. 5, pp. 518–527, 2008.

[34] A. Hubner, D. J. Mulholland, C. L. Standen et al., “JNK and PTEN cooperatively control the development of invasive adenocarcinoma of the prostate,” Proceedings of the National Academy of Sciences of the United States of America, vol. 109, no. 30, pp. 12046–12051, 2012.

[35] M. Enomoto and T. Igaki, “Src controls tumorigenesis via JNK-dependent regulation of the Hippo pathway in Drosophila,” EMBO Reports, vol. 14, no. 1, pp. 65–72, 2013.

[36] J.-C. Chen, B.-B. Lin, H.-W. Hu et al., “NGF accelerates cutaneous wound healing by promoting the migration of dermal fibroblasts via the PI3K/Akt-Rac1-JNK and ERK pathways,” BioMed Research International, vol. 2014, Article ID 547187, 13 pages, 2014.

[37] H.-J. Byun, B.-R. Kim, R. Yoo, S.-Y. Park, and S. B. Rho, “SMEK1 enhances gemcitabine anti-cancer activity through inhibition of phosphorylation of Akt/mTOR,” Apoptosis, vol. 17, no. 10, pp. 1095–1103, 2012.