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Original Article

Ebi, a *Drosophila* homologue of TBL1, regulates the balance between cellular defense responses and neuronal survival

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Abstract: Transducin β-like 1 (TBL1), a transcriptional co-repressor complex, is a causative factor for late-onset hearing impairments. Transcriptional co-repressor complexes play pivotal roles in gene expression by making a complex with divergent transcription factors. However, it remained to be clarified how co-repressor complex regulates cellular survival. We herein demonstrated that *ebi,* a *Drosophila* homologue of TBL1, suppressed photoreceptor cell degeneration in the presence of excessive innate immune signaling. We also showed that the balance between NF-κB and AP-1 is a key component of cellular survival under stress conditions. Given that Ebi plays an important role in innate immune responses by regulating NF-κB activity and inhibition of apoptosis induced by associating with AP-1, it may be involved in the regulation of photoreceptor cell survival by modulating cross-talk between NF-κB and AP-1.

Keywords: Redox-dependent transcription factors, *ebi,* TBL1, AP-1, NF-kappaB, *Drosophila,* innate immune signaling

Introduction

An inflammatory response is a highly regulated physical process that is critically important for homeostasis, such as innate immune responses, tumor formation, and neuronal survival [1-3]. Therefore, the study of this regulatory system may lead to a clearer understanding of neurodegenerative diseases, which are caused by various kinds of stress signaling pathways including inflammation. One of the common features of inflammatory response signaling is the two transcription factors, NF-κB and AP-1, which are referred to as redox-sensitive transcription factors [4, 5]. The activation of these transcription factors leads to the transcriptional induction of several target genes responsible for cell survival, differentiation, and death [6]. An increasing number of studies have indicated that cross-talk between NF-κB and AP-1 exists in immunological processes [7, 8]. A previous study reported that the binding consensus sequences of NF-κB and AP-1 were commonly observed at the promoter region of the target genes of inflammation and innate immune responses [9]. These findings suggest that one of the mechanisms underlying cross-talk is mediated by *cis*-regulatory elements at the promoter region of the target genes [9].

A large number of studies have shown that co-repressor complexes play pivotal roles in the target genes expression by associating with NF-κB and AP-1 [10, 11]. Transcriptional co-repressors regulate alternative cellular processes, such as cell cycle progression, inflammation, DNA repair responses, and programmed cell death [12, 13]. Transducin beta-like protein 1 (TBL1) and TBL1-related protein (TBLR1), two closely related F-box/WD-40-containing factors, are core components of the nuclear receptor co-repressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) co-repressor complexes [14, 15].

TBL1 has been identified as a causative factor for late-onset deafness disease, called *ocular albinism with late-onset sensorineural deafness* (OASD), which causes deafness in the fourth or fifth decade of life [16]. Sensory cell

survival is considered to be affected by mutations in TBL1; however, the molecular mechanisms responsible for this disease have not yet been clarified. A previous study revealed that the *Drosophila* homologue of TBL1, called ebi, was required for sensory neural survival [17]. Ebi appears to associate with AP-1 as well as SMRTER, a *Drosophila* homologue of the N-CoR/SMRT co-repressor complex [17-19], and represses the expression of pro-apoptotic genes, which are the target molecules of AP-1 [17]. Recent evidence suggests that Ebi also functions in innate immune response signaling, and is recruited to the promoter region of AMPs, which are the target genes of NF-κB [20]. Since *Drosophila* innate immune response signaling is similar to mammalian inflammation signaling [1], the regulation of NF-κB and AP-1 may be evolutionally conserved. The results of the present study indicate the existence of cross-talk between NF-κB and AP-1 in *Drosophila*, and also that *ebi* inhibits photoreceptor cell degeneration in the presence of excessive innate immune signaling. Therefore, Ebi is required for sensory cell survival due to its regulation of the output of NFκB and AP-1.

Material and methods

Fly stocks

The following stocks were used in the present study: Oregon-R as the wild type, *GMR-ebiΔC* [17, 21]; y^1w^{1118}; imd^1 [22]; *GMR-Gal4* [23]; *UAS-imd*, *UAS-RelN,* and *Cg-Gal4* [24] were used. *Trip-ebi* (*ebi RNAi*) was obtained from the Bloomington stock center. egr^{GS1226} was obtained from the Kyoto stock center.

Acridine orange staining

Acridine orange (AO) staining was performed as described before [25]. Briefly, eye antennal discs were dissected in PBS and incubated in 1.6 μM AO solution in *Drosophila* Ringer [26]. Samples were mounted in Vectashield (Vector).

Histochemistry

Plastic eye sections were obtained as described before [21]. Briefly, dissected eyes were fixed in 2.5% (v/v) glutaraldehyde and decalcified at 4°C for 3 d in 5 mM EDTA and 0.1 M phosphate (pH=7.4). Tissues were then fixed with 1% (w/v) OsO_4 (Wako) in phosphate-buffered saline at room temperature for 1 h. Tissues were washed with PBS for 2 h with the wash solution being changed every 15 min and then dehydrated as follows: once for 15 min in 50% (v/v) ethanol, twice for 30 min in 70% (v/v) ethanol, and once for 30 min in 85% (v/v) ethanol. After being treated with propylene oxide (Nisshin EM Co., Ltd), tissues were embedded in Epon 812 resin (TAAB Laboratories Equipment Ltd) and incubated at 60°C for 48 hrs. Toluidine Blue staining was performed after sectioning.

Statistical analysis

For statistical analyses, we used Microsoft Excel macro program (Microsoft). The significance level was set at $p<0.01$.

Results

Cross-talk between NF-κB and AP-1 in photoreceptor cell survival

Previous findings on immunological processes suggest the existence of cross-talk between NF-κB and AP-1; however, few studies have examined this in the neural system [7, 8]. In order to confirm the presence of an interaction between NF-κB and AP-1 in photoreceptor neurons, we analyzed the effects of *eiger* (*egr*), a *Drosophila* homolog of tumor necrosis factor alpha (TNF-α) at the induction of apoptosis. The overexpression of *egr* induced the activation of Jun N-terminal kinase (JNK) and AP-1 and caused apoptosis in many tissues, including eye discs (**Figure 1B**, **1F** versus **1A**, **1E**) [27]. We introduced the active form of Relish (a *Drosophila* NF-κB) into the eye imaginal discs and examined the fate of photoreceptor cells [24]. Although the activation of *Relish* itself did not induce cell death (**Figure 1C**, **1G**), it partially suppressed *egr*-induced apoptosis and weakened the eye phenotype caused by the overexpression of *egr* (**Figure 1D**, **1H** versus **1B**, **1F**) [27]. These results suggest the existence of relation between NF-κB and AP-1 in *Drosophila* photoreceptor cells and that the reciprocal regulation of these transcription factors may determine apoptotic induction during eye development.

Excessive innate immune signaling does not induce neural degeneration

The ommatidium in compound eyes was well arranged under normal conditions (**Figure 2A**, **2E**). We previously reported that *ebi* is required

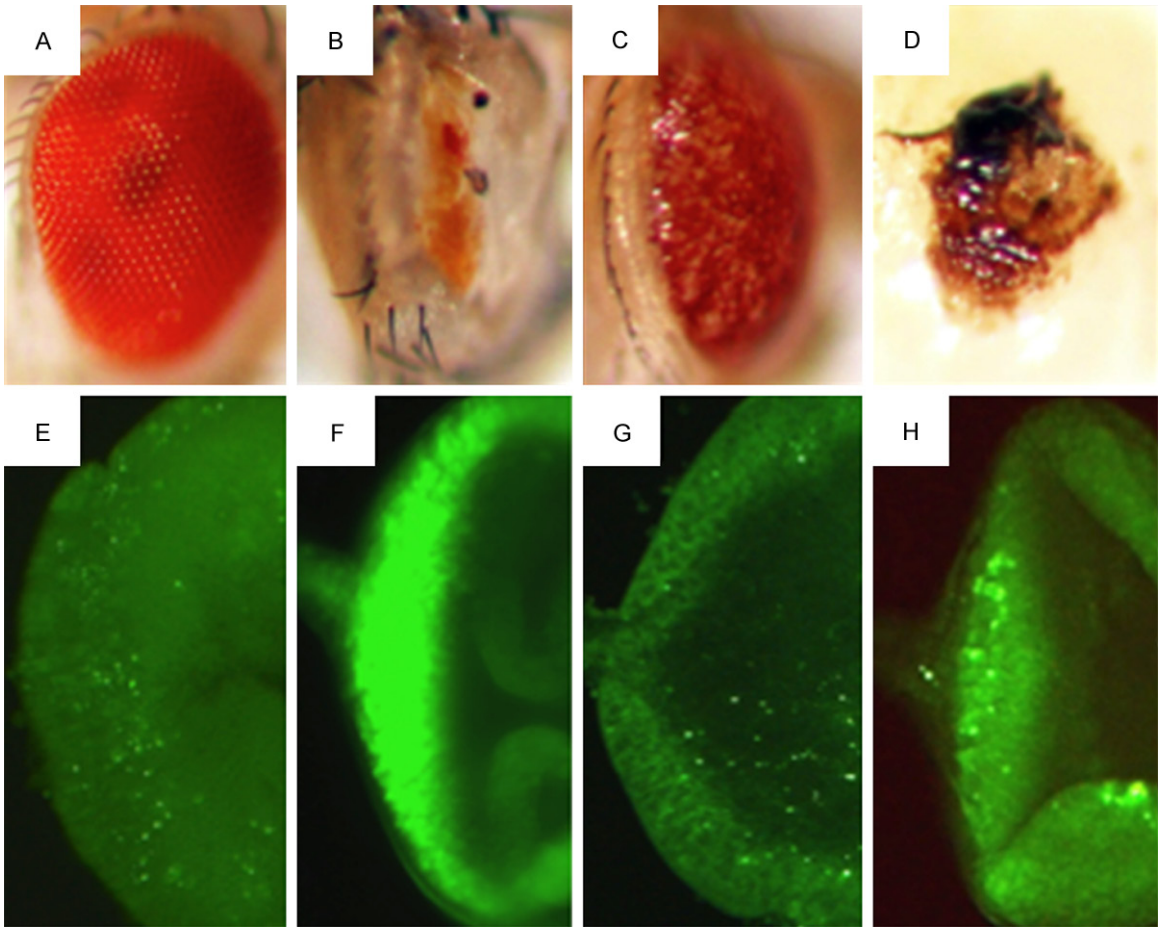

Figure 1. *Relish* activation partially suppressed *TNF*-α-induced apoptosis. (A-D) The ommatidium of each genotype and (E-H) acridine orange staining. (A, E) Wild type. (B, F) *GMR-Gal4/+; egr*^{GS1226}*/+*. *TNF*-α overexpression caused the small eye phenotype (B) and induced massive apoptosis (F). (C, G) *GMR-Gal4/+; UAS-RelN/+*. Although the overexpression of *Relish* caused the rough eye phenotype (C), apoptotic cells were not observed (G). (D, H) *GMR-Gal4/+; egr*^{GS1226}*/UAS-RelN*. *RelN* expression partially suppressed the *TNF*-α-induced small eye phenotype (D) and apoptotic cells (H).

for the survival of photoreceptor neurons through its repression of AP-1-target genes, including apoptosis-related genes in *Drosophila* (**Figure 2B**, **2F**) [17]. We also recently showed that *ebi* plays a crucial role in cellular defense responses due to its activation of NF-κB target genes under innate immune responses [20]. These findings suggest that the balance between NF-κB and AP-1 determines the expression level of the target genes responsible for cell survival, differentiation, and death, and co-repressor molecules including Ebi may also play an important role in this interaction. In order to investigate the connection among them, we focused on *imd* signaling because NF-κB and AP-1 are controlled by the signaling cascade consisting of Imd and Tak1 [28]. At first, we have observed genetic interaction among those molecules in *Drosophila* eye. We found that *imd* alone only induced minor defects in ommatidial morphology and did not cause late-onset retinal degeneration (**Figure 2C**, **2G**). Although the overexpression of the dominant negative form of *ebi* (*ebiΔC*) caused late-onset (4-5 weeks after eclosion) retinal degeneration (**Figure 2F**), which is consistent with previous findings [17], we confirmed that morphological defects caused by the overexpression of *ebiΔC* was not enhanced by the overexpression of *imd* (**Figure 2D** versus **2B**, **2C**).

Reduction of ebi caused retinal degeneration

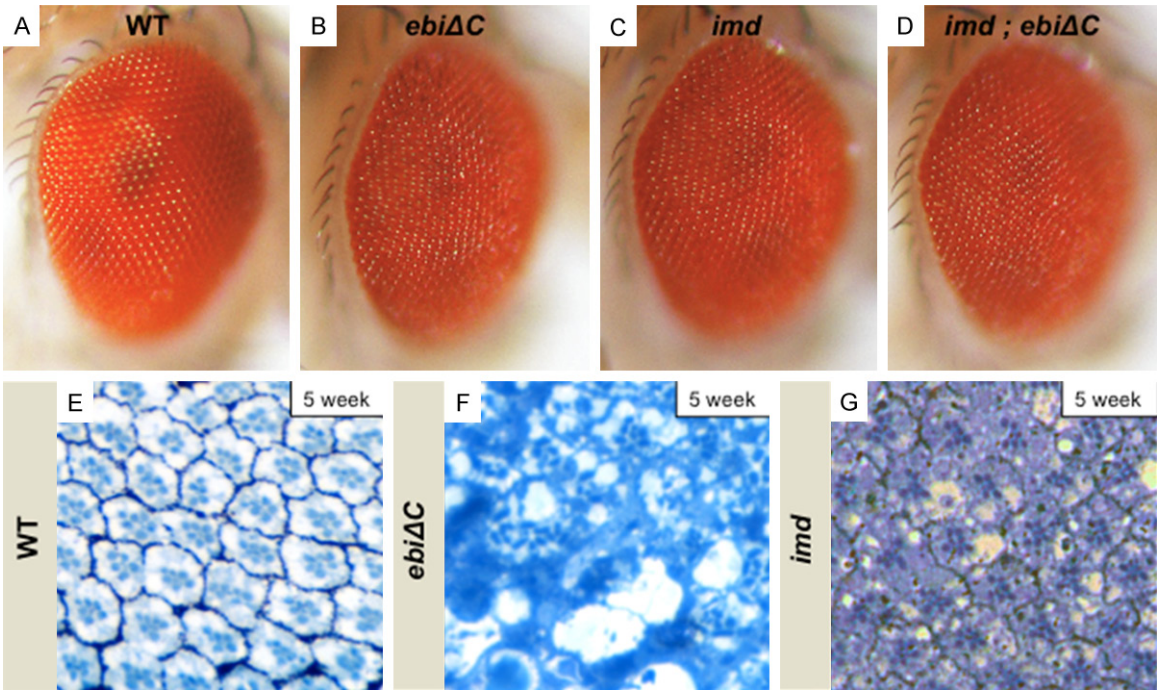

Figure 2. Imd overproduction did not induce apoptosis in photoreceptor cells. (A-D) Adult eye morphologies. Relative to the wild type (WT) (A), *GMR-Gal4>ebiΔC*: ebiΔC, a dominant negative form of Ebi, did not show morphological defects (B). The overexpression of *imd* (*GMR>imd*) (C) or *imd-ebiΔC* double Tg flies (*GMR-Gal4>imd/ebiΔC*) (D) did not induce visible morphological defects. (E-G) Eye sectioning from each genotype 5 weeks after hatching. The morphology of the WT was normal (E). ebiΔC (*Gal4>ebiΔC*) caused retinal degeneration (F), whereas the overproduction of *Imd* (*GMR>imd*) did not (G).

in the presence of excessive innate immune signaling

These results are of interest given that *imd* signaling activates NF-κB as well as AP-1 [28] and enhanced *imd* signaling has been shown to result in greater sensitivity to stress conditions [29]. We hypothesized that the activation of NF-κB and AP-1 contributes to the mild phenotype, and an imbalance between NF-κB and AP-1 leads to a challenge for cellular survival. In order to confirm this, we attempted to elucidate the genetic interaction between *ebi* and *imd*. Although the dominant negative form of Ebi (*ebiΔC*) caused retinal degeneration 5 weeks after eclosion (**Figure 2F**) [17], photoreceptor cells still remained 1 week after eclosion, as reported previously (**Figure 3A**, **3B**) [17]. Under these conditions, overexpression of *ebiΔC* and *IMD* in the eye, caused a synergistic effect: the degeneration rate of cells were increased significantly only in a week after eclosion (**Figure 3C-F**). But external morphology of eyes remained relatively normal (**Figure 2D**). These results suggest that the balance between NF-κB and AP-1 on *imd* signaling plays an important role in photoreceptor cell survival, and co-repressor complex including Ebi may be involved in the regulation of this balance.

Discussion

IMD and its mammalian counterpart, *RIP*, mediate several aspects of stress responses in addition to innate immune responses; increasing the signaling activity of these molecules results in greater sensitivity to stress conditions, such as ROS or UV irradiation, and a high incidence of apoptotic cell death [30, 31]. We previously demonstrated that *ebi* is involved in innate immune responses as well as the survival of photoreceptor neurons under stress conditions [17, 20]. In the present study, we found that the activation of IMD induced severe retinal degeneration when *ebi* activity was reduced. These results indicate that *IMD* signaling is involved in apoptotic signaling events, and requirements for AMPs may differ in each organ. Since *ebi* has distinct roles in innate immune signaling in different organs, it may

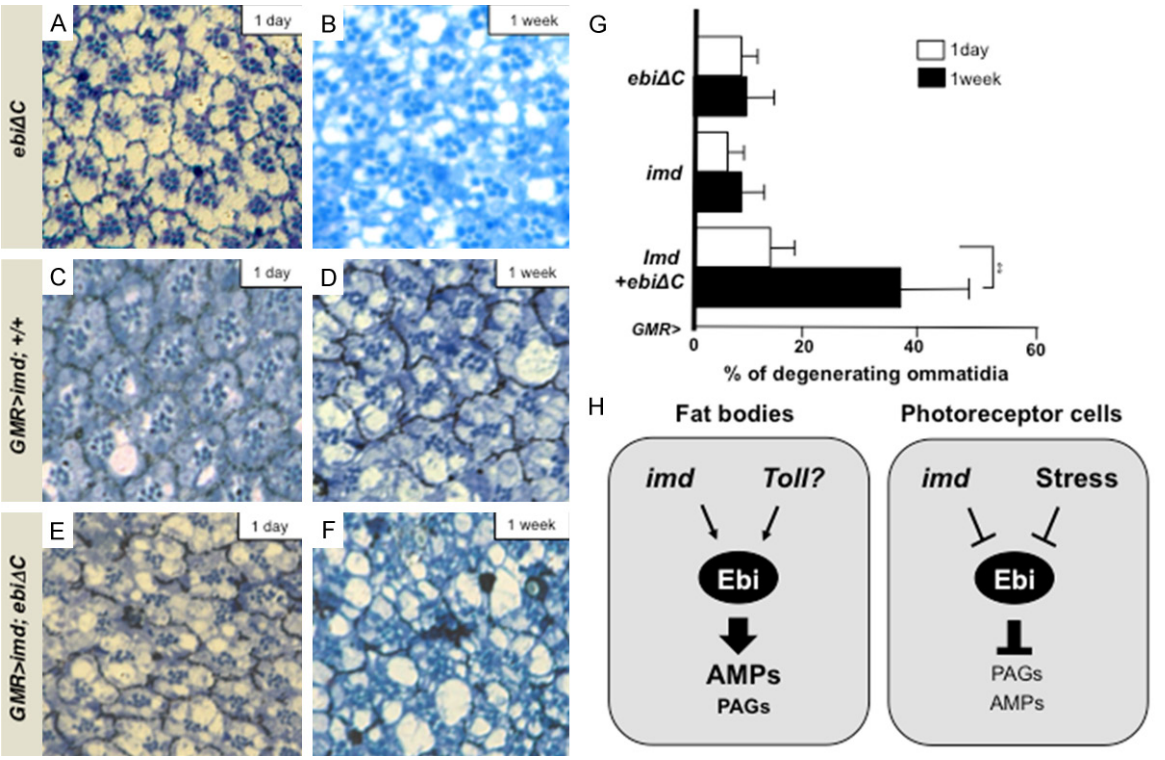

Figure 3. *Ebi* inhibited photoreceptor cell degeneration induced by *imd* overexpression. (A-F) Eye sections from each genotype. (A, B) *GMR-ebiΔC/+*. (*ebiΔC*) ommatidia 1 day (A) or 1 week (B) after eclosion. (C, D) *GMR-Gal4/+; UAS-imd/+* (*GMR-imd; +/+*) ommatidia 1 day (C) or 1 week (D) after eclosion. (E, F) *GMR-Gal4/+; UAS-imd/GMR-ebiΔC.* (*GMR-imd; ebiΔC*) ommatidia 1 day (E) or 1 week (F) after eclosion. (G) The percentage of ommatidia with degenerating photoreceptor cells is shown. 1 day and 1 week represents the numbers of days after eclosion. Data are the mean ± SEM. **P<0.01 (H) A model of *ebi* activity regulating photoreceptor cell survival under *IMD* signaling. In the innate immune system (fat body), *ebi* functions as a positive regulator for the expression of AMPs (left). In the non-innate immune system (photoreceptor cells), Ebi is required to repress the expression of pro-apoptotic genes (PAGs) (right).

contribute to the balance between the survival and death of sensory cells by modulating the expression of AMPs and PAGs (**Figure 3H**).

Cross-talk has been suggested between NF-κB and AP-1, and this interaction may be involved in the regulation of cellular survival. Innate immune signaling may regulate cellular survival through redox-sensitive transcription factors. Co-repressor complexes play important roles in this regulatory system. Notably, TBL1, a human homologue of Ebi, has been identified as a causative factor of *ocular albinism with late-onset sensorineural deafness* (OASD); patients with OASD lose their auditory ability in the fourth or fifth decade of life [16]. Since AP-1 and NF-κB are considered to play vital roles in cochlea auditory hair cells [32, 33], we predict that TBL1, in vertebrates, has an analogous role to protect sensory neurons from stress-induced apoptosis in order to facilitate the long-term functioning of auditory neurons. A clearer understanding of the molecular mechanisms by which *ebi* shows distinct functions in different tissues may lead to the biological significance of cross-talk between NF-κB and AP-1 being elucidated.

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Disclosure of conflict of interest

None.

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