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Regulatory Roles of JNK in Programmed Cell Death

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Programmed cell death or apoptosis is the regulatory mechanism for removing unneeded cells during animal development and in tissue homeostasis. Perturbation of the cell death mechanisms leads to various disorders, including neurodegenerative diseases, immunodeficiency diseases, and tumors. c-Jun N-terminal kinase (JNK) has crucial roles in the regulation of cell death in response to many stimuli. Since JNK is highly conserved from yeast to mammals, genetic studies using model animals are helpful in understanding the principal cell death mechanisms regulated by JNK. For example, loss-of-function studies using the targeted disruption of murine genes have established the genetic framework of the mechanisms of the cell death induced by UV radiation. Also, in *Drosophila*, many cell death-related genes have been identified by genetics. Genetic studies of JNK-dependent cell death mechanisms should shed light on the regulation of both physiological and pathological cell death.

Key words: *Drosophila*, Eiger, JNK, TNF, Wengen.

In physiological and non-physiological conditions, cells are continually exposed to a variety of stimuli, and they respond to each stimulus with strictly regulated inter- and intracellular signaling mechanisms. Mitogen-activated protein kinases (MAPK) play crucial roles in these responses. In higher organisms, at least three MAPK family proteins are involved in stress responses. These proteins are extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK), which is also known as stress-activated protein kinase (SAPK). JNK binds to the N–terminus of the transcription factor c-Jun (*1*, *2*) and activates it by phosphorylating serines 63 and 73 (*3–5*). In addition to c-Jun, JNK also phosphorylates activating transcription factor-2 (ATF2, also designated CRE-BP-1) and other Jun-family proteins that are involved in the AP-1 transcription factor complex (*3–7*). Fas-L (*8*, *9*) or cell death-inducing BH3-only proteins, such as Bim (*10*, *11*) and Dp5 (*11*), are induced downstream of the JNK signal, suggesting the importance of JNK-mediated transcriptional regulation of cell death.

The involvement of JNK in the control of cell death

The role of JNK in cell death signaling was first elucidated in the neuronal cell death induced by neurotrophic factor withdrawal (*12*). Once rat pheochromocytoma PC-12 cells had differentiated in the presence of nerve growth factor (NGF), the withdrawal of NGF caused the activation of JNK and subsequent cell death. The magnitude of the activation of JNK in this response was comparable to that observed when PC-12 cells were exposed to UV or heat shock (*13*). In mammals, JNK proteins are encoded by three genes. *JNK1* and *JNK2* are expressed almost ubiquitously, while *JNK3* is expressed mainly in the nervous system and to a lesser extent in the heart and testis (*6*, *14*). These genes are alternatively spliced to create at least ten JNK isoforms (*6*). *JNK1*, *JNK2*, and *JNK3* knockout mice develop normally and are indistinguishable from wild type mice. Upon closer examination, *JNK1* knockout mice showed a reduction in the delayed-type hypersensitivity in response to pathogens, which was associated with a T-cell defect (*15*, *16*); and *JNK2*-deficient mice had impaired differentiation of naive $CD4^+$ helper T (Th) cells into effector Th1 cells, but not into Th2 cells (*17*). *JNK1/2* double-knockout mice are embryonic lethal and show severe dysregulation in the control of cell death in the hindbrain and forebrain (*18*, *19*). *JNK3*-deficient mice show increased resistance to kainic acid-induced seizures and cell death of hippocampal neurons (*20*), and these phenomena are not observed in the *JNK1* or *JNK2* knockout mice (*20*). Similar results to those found in the *JNK3*-deficient mice are observed in mice with a germline mutation in which phosphorylation sites of c-Jun by JNK are replaced with alanines. These mutant mice show severe defects in the neuronal cell death induced by kainic acid (*21*). *JNK3* mutant mice also show a severe defect in the phosphorylation of c-Jun and the transcriptional activity of the AP-1 transcription factor complex (*20*). These results strongly suggest that JNK and c-Jun transduce the cell death signal through transcription-dependent machinery, at least in neurons.

JNK-dependent transcriptional regulation of cell death regulators was also observed *in vitro*. When sympathetic neurons were cultured in the NGF-deprived condition, the transcriptional upregulation of Bim, a member of the cell death-inducing BH3-only protein family, was observed, and this upregulation was inhibited by the expression of a dominant-negative c-Jun (*10*). Another BH3-only protein, Dp5, the mouse ortholog of the human Harakiri (Hrk), was also induced in a JNK-dependent manner in the cerebellar granule neurons *in vitro*, after NGF or potassium was decreased (*11*). The transcriptional activation of Fas-L was also observed when Jurkat

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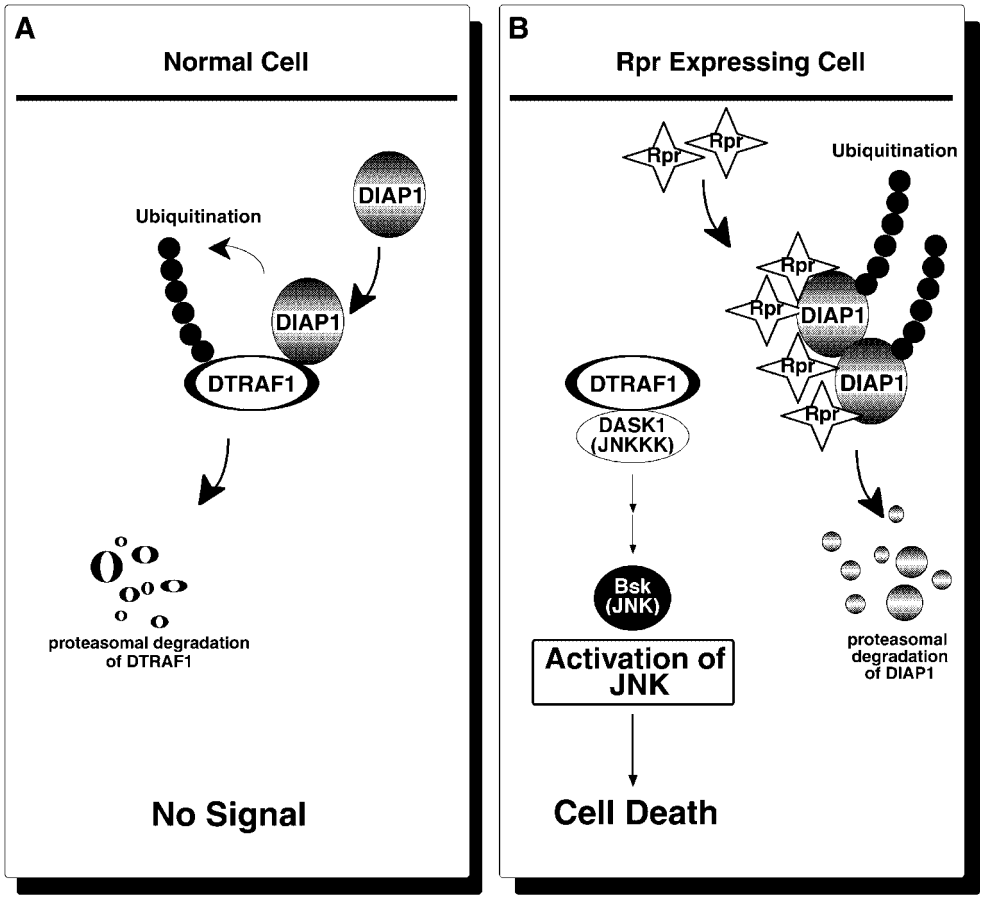

Fig. 1. **A model for the involvement of JNK in the regulation of cell death by the ubiquitin-proteasome system.** (A) Under normal conditions, DTRAF1 is degraded by the ubiquitin-proteasome system caused by the binding of DIAP1 and subsequent polyubiquitination. (B) When Reaper (Rpr) is transiently expressed in the cells, it binds to DIAP1 and promotes the self-ubiquitination of DIAP1, followed by the polyubiquitination and proteasomal degradation. The degradation of DIAP1 leads to the stabilization of DTRAF1 and induces the sequential activation of the *Drosophila* JNK pathway and cell death (*34*). DIAP1, *Drosophila* inhibitor of apoptosis protein 1; DTRAF1, *Drosophila* tumor necrosis factor-associated factor 1; DASK1, *Drosophila* apoptosis signal-regulating kinase 1

cells were stimulated with UV radiation (*8*, *9*). This transcriptional activation was inhibited by the transient expression of the catalytically inactive MEKK1 (JNKKK) (*8*), suggesting the JNK-dependent transcription of Fas-L.

JNK-mediated cell-death signaling

The genetic framework of UV-induced cell death has been studied extensively using embryonic fibroblasts from various knockout mice. UV-induced cell death was suppressed in cells derived from cytochrome-$c^{-/-}$ (Cyt-$c^{-/-}$) (*22*), Apaf-$1^{-/-}$ (*23*), Caspase $9^{-/-}$ (*24*), Caspase $3^{-/-}$ (*25*), and JNK$1^{-/-}$JNK$2^{-/-}$ (*26*) mice. The absence of JNK resulted in the failure of mitochondria to release Cyt-c when the cells were exposed to UV radiation, and cell death was suppressed (*26*). Furthermore, when the constitutively active form of apoptosis signal-regulating kinase 1 (ASK1; JNKKK) was transiently expressed *in vitro*, Cyt-c was released from the mitochondria (*27*). These results demonstrate that JNK is likely to be involved in the mitochondrial apoptotic pathway in some stress responses.

In contrast, the signaling mechanisms that connect the activation of JNK and the induction of cell death are still unclear, for several reasons. First, although JNK was reported to activate the transcriptional activity of the AP-1 transcription factor complex, new gene transcription was not required for UV-induced, JNK-dependent cell death (*26*). In brief, when primary embryonic fibroblasts (MEFs) from wild type embryos were exposed to UV radiation, neither the inhibition of protein synthesis (by cyclohexamide) nor that of RNA synthesis (by actinomycin-D) suppressed cell death, whereas JNK$1^{-/-}$JNK$2^{-/-}$ derived MEFs were resistant to the UV radiation. Second, anti-apoptotic Bcl-2 family members such as Bcl-2 and Bcl-X_L, which are known to regulate the mitochondrial membrane potential in mitochondria-related cell death mechanisms, are phosphorylated by JNK *in vitro*, and this phosphorylation suppresses their anti-apoptotic function (*28*, *29*). However, the phosphorylation of the same site has been suggested to be anti-apoptotic rather than pro-apoptotic (*30*). Furthermore, this phosphorylation has also been hypothesized to control the ubiquitin-dependent degradation of Bcl-2 (*31*).

Another reported candidate for the regulation by JNK is p53. Phosphorylation of a single site, serine 34 of the mouse p53, by JNK was observed when cells were treated with UV (*32*). However, JNK is not required for the UV-induced accumulation of p53, given that a similar accu-

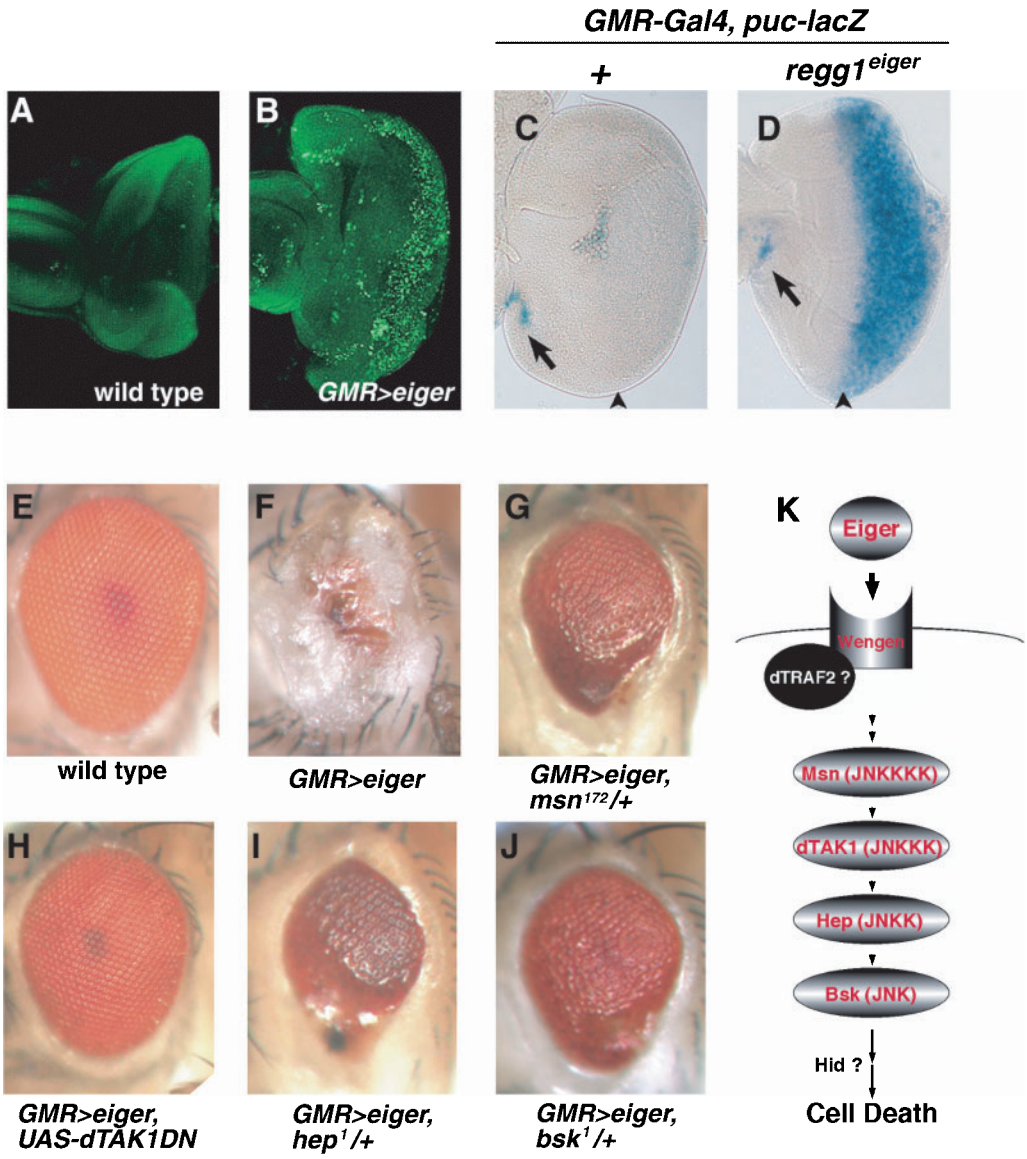

Fig. 2. **Genetic interaction of Eiger with components of the *Drosophila* JNK pathway.** (A and B) Excessive cell death was detected by acridine orange staining in the 3rd instar larval eye imaginal discs when Eiger was missexpressed driven by *GMR-Gal4* driver (*UAS-eiger*^{12}/*GMR-GAL4* as shown in (B)) compared with the wild type (A). (C and D) X-gal staining of the eye imaginal disc in the *puc-LacZ* genetic background mimicked the activation of the *Drosophila* JNK in the region posterior to the morphogenetic furrow (arrowhead) when Eiger was missexpressed in the developing eye imaginal discs using *GMR-Gal4* driver (D) compared with the control eye imaginal disc (C). The JNK activities in the disc margin, where *eiger* was not detected even in the wild type disc, were not affected (arrow). Genotypes are *GMR-Gal4*/+; *puc*^{E69}/+ (C), and *GMR-Gal4*/*regg1*^{eiger}; *puc*^{E69}/+ (D). (E-J) The downregulation of components of the *Drosophila* JNK pathway, such as *Drosophila* JNKKKK (Msn; G), JNKKK (dTAK1; H), JNKK (Hep; I), JNK (Bsk; J), suppressed the Eiger-induced small-eye phenotype. Light microscopy micrograms of wild type (E), *regg1*^{GS9830}/+; *GMR-Gal4*/+ (F), *regg1*^{GS9830}/+; *GMR-Gal4*/*msn*^{172} (G), *regg1*^{GS9830}/*UAS-dTAK1-DN*; *GMR-Gal4*/+ (H), *hep*^{1}/+; *regg1*^{GS9830}/+; *GMR-Gal4*/+ (I), *regg1*^{GS9830}/*bsk*^{1}; *GMR-Gal4*/+ (J) flies are shown. Msn, Misshapen; Hep, Hemipterus; Bsk, Basket. (K) A model for the cell death signal triggered by Eiger. In addition to the components of the *Drosophila* JNK pathway, it has been hypothesized that dTRAF2 (*42*) and Hid (*38*) should also be involved in the Eiger-induced cell death signal.

mulation of p53 was observed in response to UV irradiation in wild type and JNK1$^{-/-}$JNK2$^{-/-}$ MEFs (*26*). JNK also phosphorylates c-Myc at Ser-62 and Ser-71 when HeLa cells are treated with UV (*33*).

JNK-dependent cell death in *Drosophila*

In contrast to the diversity of the mammalian cell death signals, invertebrates have a simpler genetic architecture for regulating the cell death signals. Cell death mechanisms mediated by the JNK pathway have also been studied in *Drosophila melanogaster* (fruit fly).

Drosophila has been one of the most useful tools for investigating evolutionarily conserved cell death mechanisms. In contrast to mammals, only one *JNK* gene, *basket (bsk)*, has been found in the *Drosophila* genome. In *Drosophila*, JNK is involved in several cell death mechanisms. When one of the cell death triggers in *Drosophila*, Reaper (Rpr), was missexpressed in the *Drosophila* com-

pound eye (*GMR-rpr*), severe reduction of eye size was observed with massive cell death. The Rpr-induced small-eye phenotype is partially suppressed by the downregulation of *bsk*. *Drosophila* tumor-necrosis factor receptor-associated factor 1 (DTRAF1) and *Drosophila* apoptosis signal-regulating kinase 1 (DASK1; JNKKK) have been reported to be involved in the Rpr-induced, JNK-mediated cell death signal (*34*) (Fig. 1). The downregulation of DTRAF1 or the overexpression of a dominant-negative form of DASK1 suppressed the Rpr-induced small eye size. When DTRAF1 was transiently expressed in the *Drosophila* S2 cells, the activation of DASK1 and JNK was observed to induce cell death. At the same time, Rpr appeared to modulate the activation of the *Drosophila* JNK pathway through the degradation of the *Drosophila* inhibitor-of-apoptosis protein 1 (DIAP1), which negatively regulates DTRAF1 by proteasome-mediated degradation (*34*) (Fig. 1).

JNK is also involved in the regulation of developmental cell death in *Drosophila*. When the gradient of the Decapentaplegic (Dpp) or Wingless (Wg) morphogen is distorted in the developing wing, JNK is activated and cell death follows (*35*). Furthermore, when a discontinuity occurs in morphogen gradients, such as those of DPP or Wg, JNK is activated in the cells on both sides of the discontinuity, which is followed by cell death (*36*).

The *Drosophila* TNF superfamily ligand, Eiger, induces JNK-mediated cell death *in vivo*

Recently, the *Drosophila* TNF superfamily ligand Eiger was identified in a genome-wide screen to identify cell death triggers (*37*). When Eiger was misexpressed in the *Drosophila* compound eye using the GAL4/UAS system (*GMR>eiger*), it induced the reduction of eye size with massive cell death (*37*, *38*) (Fig. 2). The downregulation of *Drosophila* JNKKKK (Misshapen; Msn), JNKKK (dTAK1), JNKK (Hep), or JNK (Bsk) dramatically suppressed the Eiger-induced small-eye phenotype (Fig. 2), indicating that Eiger-induced cell death largely depends on the activation of the *Drosophila* JNK pathway. A loss-of-function mutant of *eiger* showed a significant reduction of the endogenous JNK activation in the eye imaginal discs (*37*). These phenomena indicated that Eiger functions as a physiological ligand for the *Drosophila* JNK pathway. One transcriptional target of JNK in the Eiger-induced cell death pathway appears to be *head involution defective* (Hid). Hid is another cell death regulator that is transcriptionally regulated in response to several pro-apoptotic signals (*39*), as well as post-transcriptionally regulated by the Ras-MAPK pathway, in response to survival signals (*40*). When Eiger was misexpressed in the *Drosophila* compound eye, *hid* mRNA expression was significantly upregulated in the eye imaginal disc (*38*).

Analysis of the Eiger Signal

Eiger-induced, JNK-dependent cell-death mechanisms have been studied both *in vivo* and *in vitro*. For example, a *Drosophila* TNF receptor superfamily protein, Wengen, was identified in a dominant-modifier screen using *GMR>eiger* and a series of chromosomal deficiency lines (*41*). This screen was designed to identify dominant suppressors of *GMR>eiger*, and several candidates for molecules that act downstream of Eiger were found (unpublished data, other than Wengen). This JNK-dependent cell death was also observed *in vitro*. When Eiger or Wengen was transiently expressed in *Drosophila* S2 cells, they induced cell death, and this cell death was suppressed by the co-expression of the *Drosophila* JNK phosphatase, Puckered (Puc) (*42*). The deduced amino acid sequence of Wengen showed it did not include the DEATH domain, which is necessary for the activation of caspases (*43*), nor the TRAF-binding domain, which is required for the activation of both NF-κB and JNK (*43*, *44*). However, dTRAF2, which is a *Drosophila* homolog of TRAF6, has been reported to activate the dorsal pathways (*45*, *46*) in *Drosophila*, physically interacted with Wengen *in vitro* (*42*) (Fig. 2). This result suggested that dTRAF2 could act as a downstream signal transducer of Wengen.

In addition to these findings, p53-dependent upregulation of Eiger, but not Wengen, was observed in a microarray assay, when embryos were exposed to ionizing radiation (IR) (*47*). However, IR-induced cell death was not suppressed in the wing imaginal discs of a loss-of-function mutant of *eiger* (*47*).

Concluding remarks

IL-3 withdrawal-induced cell death is promoted by the inhibition of JNK; hence, this cell death is suppressed by the expression of constitutively active JNK (*48*), suggesting the involvement of JNK in the IL3-mediated survival signal. JNK inactivates BAD, a pro-apoptotic Bcl-2 family protein, by phosphorylating BAD at threonine 201 (*48*). A role for JNK in the cell survival signal has also been predicted from the finding that *JNK1/2* double-knockout mice show increased cell death in the forebrain (*18*, *19*). The diversity of the roles of JNK in the regulation of cell fate most likely depends on the signal that is activated or the protein expression profile of the different cell types that express it. One of the most important focuses in current studies of the regulation of cell death by JNK is identifying essential targets of JNK. Genetic studies using model animals have made large contributions to identifying the downstream signal components of JNK. The analysis of these molecules should help us reconstruct the genetic framework of the cell death signal that is regulated by JNK. The elucidation of this framework would provide us with clues to understanding not only the physiological roles of JNK but also the mechanisms of JNK-mediated pathological conditions, which include tumorigenesis, neurodegenerative diseases, and other diseases.

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