

Structure-function analysis of Eiger, the *Drosophila* TNF homolog

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Dear Editor,

Tumor necrosis factor (TNF) family ligands in vertebrates are type II transmembrane proteins with functions in the regulation of immunity, bone homeostasis and more [1]. *Drosophila* expresses a single TNF homologue, Eiger [2, 3], which contains in addition to the homotrimeric C-terminal TNF homology domain (THD) an extended extracellular portion harboring potential protease cleavage sites (Figure 1A-i). One of these sites (Val 145) was identified experimentally [4]. A second potential cleavage site (Arg 211) resembling a canonical furin consensus sequence (R-K-S-R compared to R-X-R/K-R) was cleaved by a furin-like activity when expressed in mammalian 293T cells (Supplementary information, Figure S1). Eiger modulates host responses to *Salmonella* infections and resistance against extracellular pathogens in *Drosophila* [5, 6]. When expressed in the eye, it induces cell death via a well-defined pathway (Figure 1B) [2, 3, 7]. The structural requirements for Eiger's activity are however unknown.

As reported previously [3], full-length Eiger expressed in the *Drosophila* eye under the control of *GMR-Gal4* induced a severe apoptotic eye phenotype (Figure 1A-i and 1C-i). Expression of the entire extracellular domain (ecto-Eiger-60) also resulted in complete eye loss (Figure 1A-ii and 1C-iv). As anticipated, the THD that contains the receptor-binding domain in all vertebrate TNF ligands [8] was required for the activity (Figure 1A-vii and 1A-viii). However, shorter forms of soluble Eiger containing the THD alone (Figure 1A-v and 1A-vi) or corresponding to predicted processed forms (Figure 1A-iii and 1A-iv) were inactive despite protein expression, and also failed to interfere with death induced by full-length Eiger or ecto-Eiger-60 (data not shown). Moreover, Eiger

lacking the stalk also failed to induce apoptosis (Figure 1A-ix). All these data establish both the THD and the membrane-proximal portion of the stalk (sequence 60-145) as essential determinants of Eiger's activity.

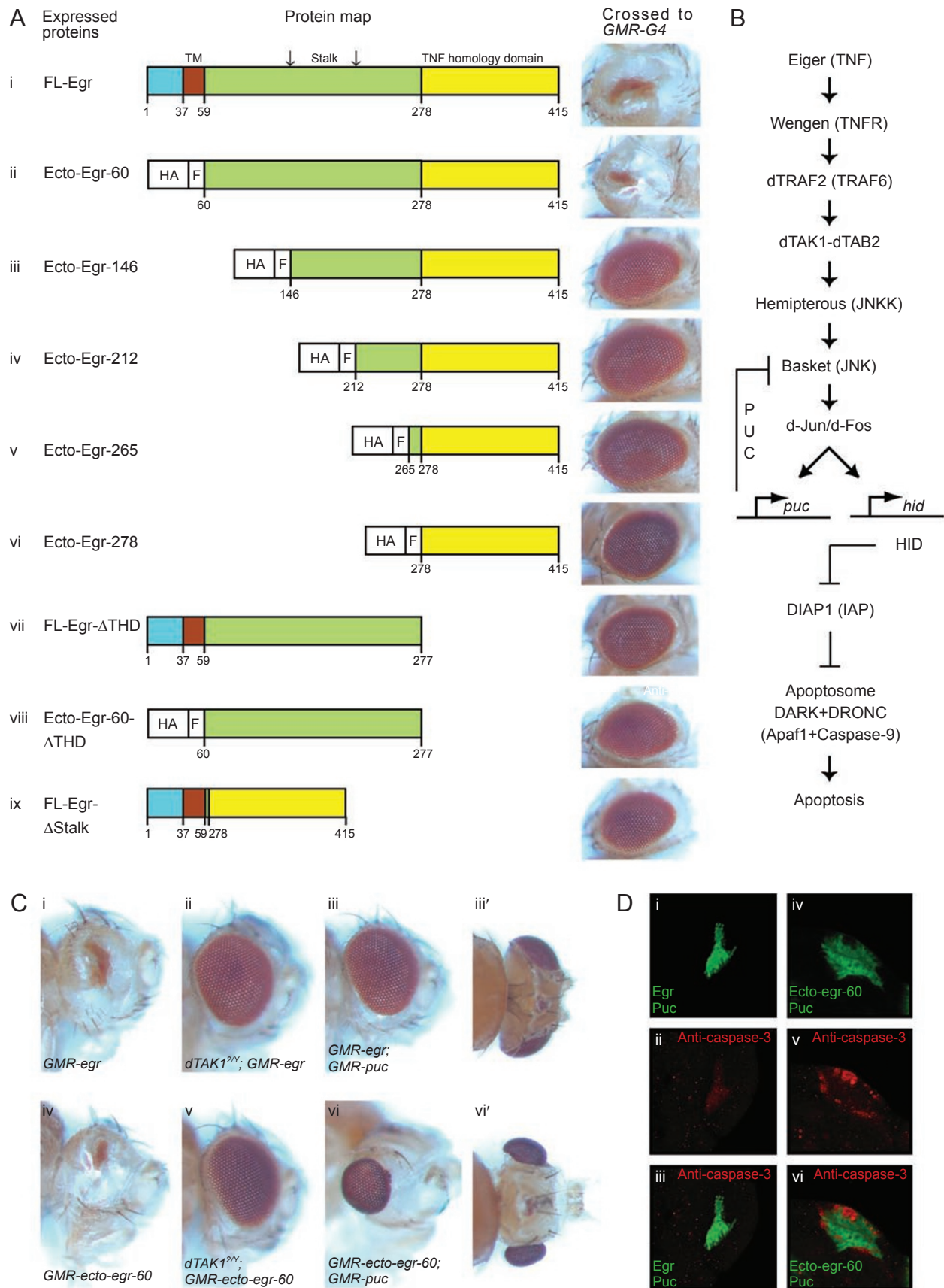
The JNK pathway mediates the apoptotic activity of Eiger [2, 3, 7] (Figure 1B). Accordingly, the apoptotic activity of ecto-Eiger-60 was totally inhibited when expressed in the eye of *dTAK1* hemizygous mutant flies (Figure 1C-v), similar to full-length Eiger expression (Figure 1C-ii). Interestingly, a novel "hanging eye" phenotype was observed when ecto-Eiger-60 was co-expressed in the eye with Puckered, a cell-autonomous inhibitor of Basket [9] (Figure 1C-vi and 1C-vi'). This phenotype, which was not observed when full-length Eiger was co-expressed with Puckered [3] (Figure 1C-iii and 1C-iii'), suggests that soluble Eiger (ecto-Eiger-60) can kill the surrounding tissue that is not protected by Puckered. This was confirmed by clonal analysis in the *Drosophila* wing imaginal disc. In this experiment, GFP-positive clones co-expressing ecto-Eiger-60 and Puckered induced caspase-3 activation in neighbouring cells where Puckered was not overexpressed (Figure 1D-iv, 1D-v and 1D-vi). In contrast, full-length Eiger failed to induce obvious activation of caspase-3 within or outside the clone when co-expressed with Puckered (Figure 1D-i, 1D-ii and 1D-iii). These results demonstrate that both full-length Eiger and ecto-Eiger-60 induce a JNK-mediated cell death, but that soluble Eiger is able to act over longer distances.

We conclude from these experiments that soluble processed forms of Eiger, if produced at all in the fly, are unlikely mediators of cell death and that the membrane-proximal portion of Eiger may contain a novel functional domain not previously described within the TNF family.

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(Supplementary information is linked to the online version of the paper on the Cell Research website.)

Figure 1 Mapping of functional domains of Eiger. **(A)** Schematic representation of expression constructs used in this study. HA, hemagglutinin signal peptide; F, Flag tag and linker; ↓, predicted cleavage sites at amino acid position 145 and 211. Numbers refer to the amino acid sequence of Eiger. The eye phenotypes generated by transgenic expression of the corresponding Eiger constructs are shown. **(B)** Schematic representation of the Eiger signalling pathway. Protein names in brackets refer to the mammalian homologue. **(C)** Long-range diffusion and death-inducing activity of soluble ecto-Eiger-60. The indicated forms of Eiger were expressed in the eye of wild-type control (i and iv) or *dTAK1*-deficient flies (ii and v) (*dTAK1*^{2/Y}, where *dTAK1*² is an inactive mutant allele of *dTAK1*), or in flies overexpressing Puckered in the eye (*UAS-puc*) (iii and vi; iii' and vi' show the eye phenotypes of iii and vi from the top). **(D)** The indicated form of Eiger was co-expressed with Puckered and GFP (by crossing *y w hs-flp; actin>CD2>Gal4 UAS-GFP* flies with flies carrying *UAS-egr*; *UAS-puc* or *UAS-ecto-egr-60*; *UAS-puc*). Clones of GFP-positive cells (in green) were analyzed in the wing imaginal disc. Cell death was detected by anti-caspase-3 staining (in red). (See supplementary information, Data S1, for the experiments described.)