Zfh1 promotes survival of a peripheral glia subtype by antagonizing a Jun N-terminal kinase-dependent apoptotic pathway

David Ohayon¹, Alexandre Pattyn¹,
Stephanie Venteo, Jean Valmier,
Patrick Carroll¹ and Alain Garces^{1,*}

INSERM U583, INM-Hopital St Eloi, 80 rue Augustin Fliche, Montpellier
Cedex, France

In *Drosophila* subperineurial glia (SPG) ensheath and insulate the nerve. SPG is under strict cell cycle and survival control because cell division or death of such a cell type would compromise the integrity of the blood-nerve barrier. The mechanisms underlying the survival of SPG remain unknown. Here, we show that the embryonic peripheral glia expresses the Zfh1 transcription factor, and in *zfh1* mutants a particular SPG subtype, ePG10, undergoes apoptosis. Our findings show that in ePG10, Zfh1 represses the pro-apoptotic RHG-motif gene *reaper* in a cell-autonomous manner. Zfh1 also blocks the activation of the Jun N-terminal kinase (JNK) pathway, and reducing or enhancing JNK signalling in *zfh1* mutants prevents or promotes ePG10 apoptosis. Our study shows a novel function for Zfh1 as an anti-apoptotic molecule and uncovers a cryptic JNK-dependent apoptotic programme in ePG10, which is normally blocked by Zfh1. We propose that, in cells such as SPG that do not undergo self-renewal and survive long periods, transcriptional control of RHG-motif gene expression together with fine tuning of JNK signalling is crucial for cell survival.

The EMBO Journal (2009) 28, 3228–3243. doi:10.1038/emboj.2009.247; Published online 10 September 2009

Subject Categories: signal transduction; differentiation & death

Keywords: apoptosis; glial cells; Jnk; *reaper/zfh1*

Introduction

In flies, as in vertebrates, the choice between cell survival and death is regulated by the balanced activities of the anti-apoptotic molecule Diap1 and the pro-apoptotic molecules of the RHG-motif family (Reaper, Head Involution Defective, Grim, Sickie and Jafrac2). Diap1 blocks activation of the intrinsic cell death machinery by binding to and neutralizing the activity of pro-apoptotic caspases (Meier *et al*, 2000; Muro *et al*, 2002; Wilson *et al*, 2002). When cells are challenged with death stimuli, the pro-apoptotic RHG proteins (White

et al, 1994; Grether *et al*, 1995; Chen *et al*, 1996), bind to Diap1, thereby liberating and activating caspases (Wang *et al*, 1999; Goyal *et al*, 2000). During embryogenesis, RHG-motif genes are expressed in highly restricted patterns that seem to correlate with programmed cell death (PCD) (White *et al*, 1994; Grether *et al*, 1995; Chen *et al*, 1996). These observations suggest that fine transcriptional control of their expression is potentially one of the major mechanisms regulating apoptosis. This issue has just begun to be addressed in studies of PCD in which cells are committed to die or to survive during tissue remodelling and establishment of final cell numbers. Thus, the *Drosophila* Hox protein Deformed controls head morphogenesis by activating localized apoptosis through direct activation of *rpr* (Lohmann *et al*, 2002). In the central nervous system (CNS), the segment-specific PCD of dMP2 neurons is regulated by the differential expression of the Hox gene *Abd-B*, which in posterior segments prevents neuronal death by repressing *reaper* and *grim* (Miguel-Aliaga and Thor, 2004). This example illustrates how, in a cell type under death pressure, survival is actively triggered through transcriptional repression of RHG-motif genes. One could thus hypothesize that, more generally, in developing tissues normally devoid of PCD a cryptic pro-apoptotic programme is present but is actively held in check notably through RHG-motif gene repression. To date, the transcriptional cascades involved in such processes remain largely unknown. Moreover, whether transcriptional regulation of the three classical RHG-motif genes (*reaper*, *hid* and *grim*) has a central role in this context is a question that remains unanswered. Strikingly, in the embryonic peripheral nervous system (PNS) no PCD seems to occur in peripheral glia (PG), and their final number is already established by embryonic stage 12 at the time when these cells become postmitotic. Moreover, in the larvae neither cell death nor further production of PG occurs (Sepp *et al*, 2000). PG appear thus as a cell type whose numbers are tightly maintained from early embryonic to late larval stages and constitute a tractable system for understanding how a particular cell type avoids apoptosis.

The *Drosophila zfh1* gene constitutes the prototype of an atypical family encoding transcription factors containing two separate krüppel-like zinc-finger clusters, and a homeodomain (Fortini *et al*, 1991). *zfh1* displays pleiotropic functions, ranging from progenitor cell specification to postmitotic precursor differentiation. Indeed, it is required for the proper differentiation of mesodermal-derived tissues such as muscle, gonads or heart (Lai *et al*, 1993; Broihier *et al*, 1998; Su *et al*, 1999). In the CNS, *zfh1* specifies the identity of motoneurons and interneurons issued from the NB7-3 lineage (Lee and Lundell, 2007). Moreover, it controls the exit of motor axons from the ventral nerve cord and has important functions in axon pathfinding of a subset of motoneurons as they grow towards their peripheral muscle targets (Garces and Thor, 2006; Layden *et al*, 2006). In addition, a recurrent feature

*Corresponding author. INSERM U583, INM-Hopital Saint Eloi, 80 rue Augustin Fliche, BP74103, 34091 Montpellier Cedex 5, France.

Tel.: +33 4 99 63 60 30; Fax: +33 4 99 63 60 20;

E-mail: alain.garces@inserm.fr

¹These authors contributed equally to this work

Received: 15 May 2009; accepted: 27 July 2009; published online: 10 September 2009

observed is that dysregulation of *zfh1* function often affects cell numbers in many tissues (Lai *et al*, 1993; Broihier *et al*, 1998; Su *et al*, 1999), although its involvement in the control of cell survival remains an open question.

In this study, we describe a cell-autonomous requirement for *zfh1* in the survival of a subset of glial cells in the *Drosophila* embryonic PNS. We show that Zfh1 is expressed by all PG and that it is required for the survival of a *gliotactin* + subperineurial glia (SPG) subtype named ePG10 (*embryonic peripheral glia 10*). Glia-specific expression of the pan-caspase inhibitor p35 in a *zfh1* mutant context, efficiently rescues ePG10 survival, migration, morphology and molecular identity. We found that *zfh1* prevents ePG10 apoptosis by repressing the expression of RHG-motif cell death activator *reaper*. Moreover, we show that in ePG10 Zfh1 blocks activation of the Jun N-terminal kinase (JNK) pathway, well known for regulating several essential developmental processes such as dorsal closure in the embryo, thorax closure, dorsal appendage formation as well as wound healing (Martin-Blanco *et al*, 1998; Zeitlinger and Bohmann, 1999; Suzanne *et al*, 2001; Ramet *et al*, 2002; Galko and Krasnow, 2004). Reduction in JNK signalling using a dominant-negative form of Basket in *zfh1* mutants is sufficient to prevent ePG10 apoptosis. Altogether these results show that a cryptic JNK apoptotic programme is indeed held in check by Zfh1 in this glial population.

Results

Zfh1 is expressed by all PG

We first characterized the expression pattern of Zfh1 within the PNS and more specifically in glial cells. PG are named according to the nomenclature of von Hilchen *et al* (2008). During stages 14–16 these cells display a highly stereotyped organization (Figure 1A and A'; see also von Hilchen *et al*, 2008). PG can be easily identified at the molecular level by the expression of the transcription factor reversed polarity (Repo), which labels all postmitotic PG from stages 12–16 (Campbell *et al*, 1994; Xiong *et al*, 1994; Halter *et al*, 1995) (Figure 1B). In the dorsal PNS, ePG11 (also known as *support cell of the dorsal bipolar dendrite cell*) and ePG10 can be reliably distinguished by their locations and characteristic morphologies. At stage 15, *Repo-Gal4::UAS-Tau-EGFP* clearly visualizes the dynamics of ePG10 migration and of its cytoplasmic processes that extend from stage 15 onward both dorsally and ventrally, along the anterior fascicle (Figure 1B).

Double –immunostaining at embryonic stages 14 and 16 for Zfh1 and Repo showed that in the abdominal hemisegments, Zfh1 is detected in all PG (Figure 1C and D). We observed the expression of Zfh1 in a Repo + cell at the position where ePG12 (also known as *lateral bipolar dendrite*) is located (asterisk in Figure 1C and D). Halter *et al* (1995) have earlier noted that this cell is positive for the neuron-specific antigen HRP and Repo. An anti-Elav antibody that labels all neuronal cells (O'Neill *et al*, 1994) confirmed Zfh1 expression in ePG12 and its exclusion from all other neurons (Figure 1E). Finally, we examined whether Zfh1 was expressed in sensory organ accessory cells using the specific marker Prospero (Campbell *et al*, 1994). No overlap between Zfh1 and Prospero could be detected in this cell type (Figure 1F). Altogether, these results show that in the embryonic PNS, Zfh1 is expressed in all PG and in ePG12, but is excluded from other neurons and accessory cells.

The ePG10 subperineurial glial cell is missing in zfh1 mutants

The glial-restricted expression pattern of Zfh1 suggested that it might have a function in glial development. We thus analysed glial cell numbers, position and morphology using combinations of two independent *zfh1* loss-of-function alleles, *zfh1*⁵ and *zfh1*^{75.26} (Lai *et al*, 1993; Broihier *et al*, 1998) and a hypomorphic allele, *zfh1*⁸⁶⁵, which is a P-element enhancer trap line with an insertion into the 5' region of the *zfh1* gene (Justice *et al*, 1995) and denoted *zfh1*^{LacZ} herein.

In the ventral region, the number and positions of PG in homozygous *zfh1*⁵, *zfh1*^{75.26} or trans-heterozygote *zfh1*⁵/*zfh1*^{75.26} mutants were similar to wild-type embryos, even if we occasionally noticed that some cells were missing and/or mislocated (Figure 2A and B). In contrast, in the dorsal region we consistently found, for all three alleles, that one glial cell was missing in the area where ePG10 and ePG11 are normally located (Figure 2A–C). To unambiguously determine the identity of this cell, we used the *J29*^{LacZ} enhancer trap for *gliotactin* (Auld *et al*, 1995) that distinguishes the subperineurial ePG10 cell (*J29*^{LacZ}+) from ePG11 (*J29*^{LacZ}–). We found that ePG11 was present in all hemisegments at all time points analysed; however, ePG10 was missing in 75% of hemisegments in *zfh1*⁵ stage 16 mutant embryos (arrowhead in Figure 2D, E and G). We also examined the status of ePG10 in *zfh1*^{75.26} homozygote and in *zfh1*⁵/*zfh1*^{75.26} trans-heterozygote mutant embryos at stage 16 and observed it to be absent in 85% (*n* = 49) and 75% (*n* = 105) of hemisegments, respectively (Figure 2G).

We next aimed to assess whether other PG were affected in *zfh1* mutants at later stages of development. We used the hypomorphic *zfh1*^{LacZ} allele that allows analysis up to larval stages because *zfh1*⁵ and *zfh1*^{75.26} mutants die soon after stage 16. In this mutant at stages 15–16 we noticed ePG10 being absent in 51% of hemisegments (*n* = 376) (Figure 2C and G) whereas other PG appear unaffected. In the first instar larval stage (stL1) mutants we found no further apparent phenotype among PG other than the lack of ePG10 (Figure 2F). Altogether, these results show that the loss of the *gliotactin* + subperineurial ePG10 in *zfh1* mutants occurs primarily between stages 13 and 16 and shows that the majority of PG differentiate grossly normally.

Zfh1 prevents ePG10 apoptosis in cell-autonomous manner

The absence of ePG10 at stage 16 in *zfh1* mutants prompted us to investigate whether this cell is never generated or progressively dies during development. In the late stage 12 of *zfh1*⁵ mutant embryos we found that ePG10 was present in 75% of hemisegments (Figure 3A, B and H) and in only 47% at stages 13–14 (data not shown; Figure 3H for quantification). This phenotype appeared even more severe by late stage 15, ePG10 being present in just 25% of the hemisegments as mentioned above (Figure 3H).

The progressive decrease in ePG10 numbers between late stages 12 and 16 raised the possibility that in *zfh1* mutants, ePG10 undergoes apoptosis. In line with this hypothesis, in *zfh1* mutants we frequently found pyknotic cells with shrunken and densely stained nuclei at the site where ePG10 is normally found (double arrowhead in Figure 3B; see also Figure 2B). In late stage 12, pyknotic ePG10 nuclei were

Figure 1 Zfh1 is expressed in all peripheral glia. (A, A') Diagrams showing the location and morphology of PG nuclei from stages 14–16 and the molecular markers expressed by these cells; adapted from von Hilchen *et al* (2008). (B) In wild-type stage 15 flat-mounted embryos, *Repo-Gal4*, *UAS-EGFP^F* show the morphology of PG. Closeup view 1 shows Repo expression in the ePG12 neuron. In the dorsal region, ePG10 migrates from a dorsal to a ventral position (compare closeup views 2 and 3). (C, D) Double labelling for Zfh1 and Repo shows that all glial cells of the PNS express Zfh1. At stage 14, the nuclei of ePG10 and ePG11 display a characteristic rounded morphology, whereas at stage 16 these cells have an elongated nucleus (compare closeup views in C and D). (E) Double staining for Zfh1 and Elav shows that, with the exception of the ePG12 neuron (asterisk), PNS neurons do not express Zfh1. Closeup views show expression of Zfh1 in the ePG12 neuron. (F) Double staining for Zfh1 and Prospero shows that Zfh1 is not expressed in PNS accessory cells. In all panels two consecutive hemisegments are shown. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: 5 ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right. The dashed line indicates the lateral edge the CNS.

found in 15% of hemisegments ($n = 96$) whereas they were never found in wild-type counterparts. To confirm that ePG10 undergoes apoptosis in *zfh1* mutants we used an antibody raised against the activated form of the human caspase-3 protein (denoted Casp3*). This antibody was shown earlier to recognize apoptotic cells in *Drosophila* tissues (Yu *et al*, 2002; Brennecke *et al*, 2003). Casp3* staining showed no cell death in PG in stages 15–16 wild-type embryos (Figure 3C). In contrast, in *zfh1⁵* mutants, Casp3* was detected in all

dorsally located Repo+ nuclear remnants, which presumably correspond to the dying ePG10 (Figure 3D). These results show that in the absence of *zfh1*, ePG10 is generated and degenerates by apoptosis.

We next aimed to determine whether *zfh1* acts postmitotically and in a cell-autonomous manner to ensure ePG10 cell survival. The fact that motor axons bundles are affected in *zfh1⁵* mutant embryos (Layden *et al*, 2006) could argue for an indirect effect of motor axons on ePG10 survival as this cell is

Figure 2 The dorsal ePG10 glial cell is missing in *zfh1* mutant. (A) In wild type, Zfh1 and Repo are co-expressed in all the glial cells of the PNS. (B) No Zfh1 protein was detected in homozygote *zfh1⁵*, indicating that this allele is a protein null. In the dorsal region of the PNS ePG10 is absent in 72 % of the hemisegments whereas ePG11 is always present. Double arrowhead indicates shrunken and densely stained nucleus at the normal position of ePG10. (C) In *zfh1^{LacZ-/-}*, ePG10 is absent in two consecutive hemisegments. (D) In wild-type embryos, *J29^{LacZ}* shows ePG10 in all hemisegments. (E) In *zfh1⁵* mutant embryos, ePG10 is rarely seen in the dorsal region of the PNS. (F) In first-stage larvae (stL1) ePG10 is frequently absent whereas other PG appear normal. Two consecutive hemisegments are shown. (G) Quantification of ePG10 cell survival at stages 15–16 in different mutant combinations compared with wild type. Of note, the rescue experiment using *UAS-zfh1* was carried out at 29°C to increase Zfh1 dosage. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right. The dashed line indicates the lateral edge the CNS.

found in close contact with the dorsally projecting *inter segmental nerve* (ISN). In contrast to *zfh1⁵* allele we found that in *zfh1^{LacZ}* hypomorphic mutants ISN motoneurons develop and project normally towards their dorsal muscle fields. Nevertheless, ePG10 still dies in this context (Figure 3E and F; see Figure 2G for quantification) supporting the idea that motoneurons axons bundles do not have a key function in its survival. We then used, on a *zfh1⁵* mutant background, the *Repo-Gal4* driver to specifically restore *zfh1* activity in

postmitotic glial cells (and not in motoneurons) (Sepp and Auld, 2003). In such embryos, the death of ePG10 was efficiently (albeit partially) rescued, ePG10 being present in 75 % of the hemisegments at stages 15–16 (*n* = 130) (Figure 3G and H). Furthermore, in this context the position and morphology of ePG10 nuclei were similar to wild type (Figure 3G). Collectively, these results show that *zfh1* acts postmitotically and cell autonomously to prevent ePG10 cell death.

Figure 3 *zfh1* is postmitotically and cell autonomously required for ePG10 survival. (A) In late stage 12 *Zfh1* is expressed in ePG10 but not in ePG11. (B) In late stage 12 *zfh1*⁵ mutants ePG10 is present or appears pyknotic (double arrowhead). (C) In wild type, no detectable Casp3* staining is seen in Repo+ cells. (D) In *zfh1*⁵ mutant embryos, Casp3* staining shows that, in the dorsal region of the most anterior hemisegment, ePG10 undergoes apoptosis. (E) In *zfh1*^{LacZ+/-} embryos, ePG10 (Repo+, βGal+) is found in close association with dorsally projecting inter segmental nerve (ISN) motor axons (Fas2+). (F) In *zfh1*^{LacZ-/-} embryos, ePG10 is absent in two hemisegments where ISN motor axons project normally. (G) Using *UAS-zfh1;repo^{Gal4}* in overall *zfh1*⁵ homozygote mutant embryos, ePG10 is present in 75% of the hemisegments and migrates towards its final ventral position. (H) Quantification of ePG10 cell survival in different mutant combinations compared with wild type. Note that the absence of ePG10 is more severe in stages 15–16 than in stages 13–14 and than late stage 12 indicating that in some hemisegments ePG10 cell death occurs mainly between late stage 13 and stage 16. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right. The dashed line indicates the lateral edge the CNS.

Loss of ePG10 in *zfh1* mutants is not due to aberrant differentiation

It could be argued that ePG10 cell death in *zfh1* mutants resulted from impaired differentiation (i.e. aberrant molecu-

lar identity and/or migratory behaviour and/or cell morphology). To test this possibility we used the *Repo-Gal4* line to express the baculoviral anti-apoptotic caspase inhibitor p35 (Hay *et al*, 1994) in glial cells, in homozygote *zfh1*⁵ mutant

embryos. This resulted in the survival of ePG10 in 88% of hemisegments ($n=110$) (Figure 4A–F). Moreover, in this allelic combination we found that (i) rescued ePG10 expresses Repo, *wingless*, *mirror* (showed by the enhancer trap lines *wg^{LacZ}* and *mirr^{LacZ}*; see Figure 4A–D) and *Gliotactin* (data not shown); (ii) the migration and positioning of ePG10 is ventral to ePG11 as in wild-type embryos (Figure 4E and F) and (iii) its specific shape, i.e. elongated nucleus oriented perpendicular to ePG11, is similar to the wild type (Figure 4E and F). In addition, use of *Mz97^{Gal4}*, which is normally expressed in ePG10, further outlines the correct molecular identity and morphology of this cell (ex-

tended cytoplasmic processes lying perpendicular to ePG11) in *zfh1* mutants (Figure 4G and H). Taken together these data show that *zfh1* prevents apoptosis of the ePG10 glial cell but is not required for other aspects of its differentiation.

Genetic interactions between *zfh1*, *Diap1* and RHG-motif genes in ePG10

As *Diap1* and RHG-motif genes are key regulators of cell survival/death, we examined their putative genetic interaction with *zfh1* for the control of ePG10 survival. Using the *th^{5C8}* enhancer trap line (a P-element LacZ insertion upstream of *Diap1*; see (Hay *et al*, 1995) we showed that

Figure 4 *zfh1* does not have an instructive function for ePG10 differentiation other than survival. (A–H) Using *UAS-p35; Repo^{Gal4}*, *zfh1⁵* ePG10 survives in 88% of hemisegments. Rescued ePG10 expresses Repo (B, D, F, H), *mirror* (from *mirr^{LacZ}*) (B), *wingless* (from *wg^{LacZ}*) (D) and *Mz97^{Gal4}* (H), similarly to wild-type counterparts. The morphology of rescued ePG10 (elongated cytoplasmic processes oriented perpendicular to ePG11) is similar to wild type (compare H and G). In stage 16 embryos, rescued ePG10 migrates ventrally as in wild type (compare E and F). In each case, only two consecutive hemisegments are shown. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right. The dashed line indicates the lateral edge the CNS.

Diap1 is expressed in ePG10 (Figure 5A and A'). This line has been reported to be a hypomorphic allele of *Diap1* and, in *th^{ISC8}/th^{ISC8}* mutant embryos, we found that ePG10 undergoes

apoptosis in 32% of the hemisegments ($n = 139$) (Figure 5B, B' and E). Moreover, over-expression of *Diap1* in PG using the allelic combination *UAS-Diap1; Repo-Gal4* in *zfh1⁵* homozy-

Figure 5 *Diap1* and *H99* are epistatic to *zfh1* function. (A, A') In *diap1^{LacZ/+}* heterozygous embryos, ePG10 and ePG11 are present and express *diap1^{LacZ}*. (B, B') In *diap1^{LacZ/-}* homozygous embryos ePG10 is absent in 32% of the hemisegments ($n = 139$) whereas ePG11 survival is not affected. The (A', B') panels are, respectively, identical to (A) and (B) but only the Repo channel is shown. (C) In overall *zfh1⁵* mutant embryos, *UAS-diap1; Repo^{Gal4}* rescues ePG10 survival. (D) In homozygote *H99, zfh1⁵* mutant embryos, ePG10 (*J29^{LacZ}+*) is present in 62% of the hemisegments showing that ePG10 cell death in *zfh1* mutants is RHG-motif genes dependant. (E) Quantification of ePG10 cell survival in different mutant combinations in stage 16 compared with wild type. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right. The dashed line indicates the lateral edge the CNS.

gous mutant embryos, led to survival of the ePG10 cell in 93% of hemisegments ($n=176$) (Figure 5C and E). In parallel we analysed the effect of the homozygous deletion *Df(3L)H99* lacking *rpr*, *hid* and *grim* (denoted *H99*) on ePG10 cell death in a *zfh1*⁵ homozygous mutant background. In *zfh1*⁵*H99* double mutants embryos we found that apoptosis of ePG10 can be prevented since ePG10 (identified by the *J29^{LacZ}*) is present in 62% of hemisegments analysed ($n=104$) (Figure 5D and E). We also quantified ePG10 cell death in *zfh1*⁵ homozygous mutant embryos that are heterozygous for the *H99* deletion and found ePG10 in 65% of hemisegments ($n=204$), showing that only one copy of the wild-type *rpr*, *hid* and *grim* genes is not sufficient to induce ePG10 cell death (Figure 5E). These results show that (i) the death of ePG10 observed in *zfh1* mutants is apoptotic in nature, confirming our earlier experiments with p35; (ii) ePG10 survival is sensitive to the level of expression/activity of Diap1 and RHG-motif genes; and (iii) Diap1 activity and *H99* deletion are epistatic to Zfh1 function.

Analysis of Diap1 and RHG-motif genes expression in *zfh1* mutants

As Zfh1 is a transcriptional regulator, these data raised the possibility that Zfh1 has an anti-apoptotic function in ePG10 by influencing Diap1 and/or RHG-motif gene expression. To test this idea, we first monitored *Diap1* expression using the *th^{15C8}* insertion in *zfh1*⁵ mutants at late stage 12, before ePG10 cell death, and found normal *Diap1* expression (Supplementary Figure S2F, G). In stage 16 homozygote *zfh1*⁵ mutant embryos in which ePG10 survival was rescued using *UAS-p35*, *Repo-Gal4* we also found normal *Diap1* expression (Figure 6A and B). Thus, despite the role of *Diap1* in ePG10 survival this observation indicates that in *zfh1* mutants, ePG10 cell death is not a consequence of downregulation of *Diap1*. We also confirmed this at the protein level by using an antibody against Diap1 (data not shown). We then studied the status of RHG-motif gene expression in the same genetic background. By double immunostaining we found that neither Hid nor Grim expression is upregulated in ePG10 (Supplementary Figure S1 A–D'). Since to date no Rpr antibody suitable for immunostaining procedures on fixed tissues has been reported, we thus monitored *rpr* expression *in vivo* using a Gal4 P-element insertion into *rpr*, *rpr^{NP0520}* (Miguel-Aliaga and Thor, 2004) and denoted *rpr^{Gal4(520)}* herein. In wild-type embryos, *rpr^{Gal4(520)}* expression was not detected in ePG10 ($n>82$ hemisegments). In contrast, in *zfh1* mutant embryos at stages 13–14 its expression was observed in this cell in 10% of hemisegments ($n=130$). Similar results were obtained using the *rpr^{Gal4(368)}* enhancer trap (Figure 6C and D; data not shown). This result most probably reflects that ePG10 apoptosis does not occur simultaneously in each hemisegment (see Figure 3H) and highlights that each analysed embryo should only be regarded as a unique picture taken at a given time. Altogether, these results show that while the expression of *Diap1*, Hid and Grim is not altered in ePG10 in *zfh1* mutants, *rpr* is deregulated.

rpr as a major cell death executor in ePG10

The above observations indicate that *zfh1* represses *rpr* and suggests that among the three classical RHG-motif genes *rpr*

is the major cell death determinant of ePG10 in *zfh1* mutant. To confirm this hypothesis we took advantage of the availability of a hypomorphic allele of *rpr*, *rpr^{Gal4(520)}* (Miguel-Aliaga and Thor, 2004), and another uncharacterized *rpr* allele, *rpr^{Gal4(368)}*. First, we characterized the Gal4 enhancer trap allele *rpr^{Gal4(368)}* by showing that this P-element lies 335 base pairs upstream of the *rpr* start codon and 43 base pairs upstream of the *rpr^{Gal4(520)}* P-element (Figure 6E₁ and E₂). Furthermore, by western-blot analysis we have found that the level of Rpr protein is decreased by 2.5-fold in homozygote *rpr^{Gal4(368)}*-pooled embryos compared with wild type (Figure 6E₃). We then analysed *rpr^{Gal4(368)}−/−*, *zfh1*⁵*−/−* and *rpr^{Gal4(368)}+/−*, *zfh1*⁵*−/−* embryos and found ePG10 survival in 75% ($n=210$) and 64% ($n=150$) of hemisegments, respectively (Figure 6F; Supplementary Figure S2H). Similar results were obtained using the *rpr^{Gal4(520)}* allele (Figure 6F). These figures are strikingly comparable with those found using the XR38 deficiency, which removes *rpr* and *sickle* (Peterson *et al*, 2002) (Figure 6F) and the *H99* deficiency (see Figure 5E). Altogether these results highlight a key role for *rpr* in ePG10 cell death in *zfh1* mutants.

ePG10 apoptosis in *zfh1* mutants requires activation of the JNK pathway

We then aimed to determine whether the death of ePG10 in *zfh1* mutants is the consequence of the activation of known pro-apoptotic signalling pathways. Among them, we focused our attention on the JNK pathway that represents one good candidate. Indeed, molecular determinants of this pathway are known to have a major function in triggering apoptosis during development in *Drosophila* (Adachi-Yamada *et al*, 1999a; McEwen and Peifer, 2005) and in vertebrates; it has been shown to influence the survival of PG (Parkinson *et al*, 2001). In *Drosophila*, JNK pathway activity can be monitored by transcriptional activation of *puckered* (*puc*), a dual-specificity phosphatase that acts in a negative-feedback loop to regulate the JNK pathway (Martin-Blanco *et al*, 1998). *puc* enhancer traps have been widely used to monitor at the cellular level resolution JNK activity *in vivo* (e.g. Adachi-Yamada *et al*, 1999a; Tateno *et al*, 2000; Igaki *et al*, 2002). Indeed, in response to apoptotic stimuli the *pucE69* enhancer trap is induced in a JNK-dependent manner (Adachi-Yamada *et al*, 1999a). We thus first analysed the activation state of this pathway in ePG10 in wild type and *zfh1* mutant backgrounds. In wild-type late stage 12 and stage 13 embryos we found that β Gal expression from *pucE69* is low or under detectable levels in the developing ePG10 (Figure 7A and C). In contrast, in equivalent stage *zfh1* mutants, we found *pucE69* expression to be significantly increased in ePG10 (Figure 7B and D) showing that JNK pathway is potentiated in *zfh1* mutants.

We next decided to study the putative effect of this JNK pathway activation on the death of ePG10 in *zfh1* mutant embryos. First, we used *UAS-Puc* driven by *Repo-Gal4* to antagonize JNK signalling. This led to the significant survival of ePG10 in 58% of hemisegments ($n=268$) from stages 13–16 (Figure 7F; data not shown). These results support the idea that death of ePG10 in *zfh1* mutants involves, at least in part, the recruitment of the JNK signalling. Second, we took advantage of the existence of a dominant-negative form of JNK (Basket), *UAS-bsk^{DN}* (*Bsk^{DN}*) (Adachi-Yamada *et al*, 1999b), which is the most potent JNK pathway inhibitor

Figure 6 *Reaper* is necessary for ePG10 cell death in *zfh1* mutant. (A) In *diap1*^{LacZ/+} embryos ePG10 and ePG11 express βGal. (B) In *UAS-p35; Repo*^{Gal4}, *diap1*^{LacZ/+}, *zfh1*^{5-/-} embryos, we found normal βGal expression in ePG10 indicating that in this cell *zfh1* does not regulate *diap1* expression. (C) In *UAS-nlsEGFP; rpr*^{Gal4(520)/+} embryos, GFP expression is not detected in the developing Repo + cells. (D) In *UAS-p35, UAS-nlsEGFP; rpr*^{Gal4(520)/+}, *zfh1*^{5-/-} embryos, GFP expression is seen in ePG10. (E₁) Schematic view of the *rpr* gene locus. The open reading frame for Rpr is indicated in red. Locations of the two P-Gal4 element insertions *rpr*^{Gal4(368)} and *rpr*^{Gal4(520)} used in this study as mapped by the NP Consortium. Small arrows denote the position of the oligonucleotides used for the genomic DNA PCR. (E₂) PCR on genomic DNA showing that the *rpr*^{Gal4(368)} P-element lays 335 base pairs upstream of the *rpr* start codon and 43 base pairs upstream of the *rpr*^{Gal4(520)} P-element. (E₃) western-blot analysis showing that the level of Rpr protein is decreased by 2.5-fold in homozygote *rpr*^{Gal4(368)}-pooled embryos compared with wild type. (F) Quantification of ePG10 cell survival in different mutant combinations in stage 16 compared with wild type. Note that ePG10 cell death is significantly rescued using heterozygous or homozygous combinations of the *rpr*^{Gal4(368)} or the *rpr*^{Gal4(520)} hypomorphic allele of *rpr* in overall *zfh1*^{5-/-} mutant embryos. As a control, *zfh1*^{5-/-} homozygotes mutants (denoted 'reverted') obtained after removal of the *rpr*^{Gal4(368)} allele from the combined *rpr*^{Gal4(368)}, *zfh1*^{5-/-} chromosome leads to ePG10 survival in only 35% of hemisegments. These results strongly support the view that ePG10 cell death in *zfh1* mutants is primarily *rpr* dependant. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right.

available so far (Galko and Krasnow, 2004). Specific over-expression of Bsk^{DN} in glial cells, resulted in the survival of ePG10 in 83% of hemisegments (*n* = 367) compared with 35% (*n* = 81) in *zfh1* mutant embryos from stages 13 to 16 (Figure 7E and F; data not shown). Altogether these results show that in the absence of Zfh1 the JNK signalling pathway is activated in ePG10 and triggers its apoptosis. They also support the view that Zfh1 acts as a negative regulator of this signalling.

Zfh1 is capable of inhibiting apoptosis downstream of JNK activation

The earlier experiments showed that Zfh1 is epistatic to the JNK pathway. To investigate at which level(s) of the pathway (of which the core components are →Hep→Bsk→DJUN→Puc) Zfh1 may act, we took advantage of the availability of an activated form of the MAPKK Hemipterous (Hep) and denoted HepAct here. We thus over-expressed HepAct in all glial cells using *RepoGal4::UAS-HepAct* in different conditions of

Figure 7 In *zfh1* mutants JNK signalling is active in ePG10 and controls its apoptosis. (A, C) Double staining for Repo and βGal shows that in wild-type embryos *Puc^{LacZ}* expression is under detectable level (late stage 12) or low (stage 13) in ePG10. Note that higher levels of *Puc^{LacZ}* expression are detected in ePG11 cells. (B, D) In *zfh1* mutants *Puc^{LacZ}* expression was increased in ePG10 both at late stage 12 and stage 13 embryos. (E) Forced expression of Bsk^{DN} in glial cells prevents ePG10 cell death. (F) Quantification of ePG10 cell survival in different mutant combinations compared with wild type in stage 15 embryos. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right.

zfh1 gene dosage. By monitoring *pucE69* expression as a readout we noticed strong activation of the pathway in most PG, independently of the *zfh1* gene dosage condition. It is notable that in the ventral region we consistently found a pool of three or four glial cells that never displayed robust *pucE69* expression (Figure 8A–C) indicating they are not permissive to JNK pathway activation. In contrast, we found robust expression of *pucE69* in ePG10 despite the presence of Zfh1. This result suggests that the earlier shown interference of Zfh1 with the JNK pathway occurs at the level of or upstream of Hep. Surprisingly, over-expression of HepAct in wild-type embryos did not trigger massive cell death among glial cells and in ePG10 in particular. However, careful quantification showed apoptosis of ePG10 in 4% of hemisegments at stage 16 ($n = 146$) thus confirming the potential pro-apoptotic activity of this signalling (Figure 8A, B and D for quantification). This phenotype indicates that pro-survival determinants might act by blocking downstream cell death effectors triggered by this pathway. In light of the anti-apoptotic effect of Zfh1 in ePG10 we addressed whether

this molecule could fulfil such a role. To test this hypothesis we over-expressed HepAct in *zfh1*⁵ heterozygotes. This led to the death of ePG10 in 16% of hemisegments (either absence or pyknotic nuclei of ePG10) showing a four-fold increase in cell death compared with the same experiment carried out in wild-type background (Figure 8B–D).

Altogether these experiments show that Zfh1, in addition to its role as an inhibitor of the JNK pathway, can also prevent apoptosis by blocking downstream pro-apoptotic effectors triggered by JNK signalling. On the basis of our genetic evidence we propose the model presented in Figure 8E to illustrate how Zfh1 may act to prevent cell death in ePG10.

Discussion

Control of cell survival by Zfh1 family members: a general feature?

In *Drosophila*, several studies have showed that loss of *zfh1* function often leads to a reduction in cell numbers in

Figure 8 Zfh1 is able to inhibit ePG10 apoptosis downstream of JNK activation. (A–C) When HepAct is over-expressed in glial cells ePG10, ePG11, ePG12 and a subset of PG located ventrally strongly express *Puc*^{LacZ} compared with wild type. Interestingly, in each hemisegment three or four ventrally located glial cells never displayed robust *Puc*^{LacZ} expression. (D) Quantification of ePG10 cell survival when JNK signalling is activated in different conditions of *zfh1* gene dosage compared with wild type. (E) Proposed model for ePG10 survival. A *DIAP1* and *reaper*-dependant cell death mechanism in ePG10 are controlled by *zfh1* and JNK activation. Zfh1 may block transcription of one or more component(s) of the JNK signalling, including upstream actor(s), thus shutting down or maintaining a low-activated state of this pro-apoptotic pathway (grey line). Alternatively, Zfh1 may repress *rpr* independently of its control of the JNK pathway. The fact that *rpr* has been reported in other models to be able to activate its own expression by sequestering *DIAP1* and thus recruiting the JNK pathway (grey line) could explain why in *zfh1* mutants increased expression of *puckered* is observed. These two mechanisms are not exclusive as Zfh1 may be able to repress in parallel components of the JNK pathway and *rpr*. Red and green indicate pro-apoptotic and anti-apoptotic molecules, respectively. Arrowheads: ePG10; arrows: ePG11; asterisks: ePG12 neuron; brackets: five ligament cells of the chordotonal organ. Anterior is up and dorsal is to the right.

mesodermal derivatives (Lai *et al*, 1993; Moore *et al*, 1998; Su *et al*, 1999). However, it is still not known whether these phenotypes are due to aberrant cell death and/or proliferation defects. In the light of our present work it will be interesting to see whether *zfh1* also has an anti-apoptotic function in these structures. Recently, it was shown that specific inactivation of *Zfhx1b* in the mouse embryonic dorsal telencephalon leads to proliferation defects and to increased apoptosis in the developing hippocampus. In particular, apoptotic figures were found in the cortical plate in which differentiating neurons normally accumulate (Miquelajauregui *et al*, 2007). In humans, while mutations

or deletions in *ZFH1B* have been found to cause the Mowat–Wilson Syndrome, which is characterized by typical facial features, microcephaly and severe mental retardation (Mowat *et al*, 2003), nonsense and frameshift mutations in *ZFH1A* (also named *TCF8*) have been associated with *Fuchs* corneal dystrophy (Krafchak *et al*, 2005). Patients with *Fuchs*' dystrophy display a progressive loss of cells in the corneal endothelium and it has been proposed that excessive apoptosis may be an important mechanism in the pathogenesis (Borderie *et al*, 2000; Li *et al*, 2001). In addition, *ZFH1A* was shown to have a key function within a neuronal survival pathway rapidly induced in response to cortical ischemia. This

pro-survival response, initiated through the rapid induction of p63, is mediated ultimately by the transcriptional repression of a pro-apoptotic isoform of p73 by ZFH1A (Bui *et al*, 2009). These observations together with our results indicate that an anti-apoptotic role of *Zfh1* family members may be a general feature and thus have important impacts on the understanding of the function of this family of transcriptional regulators in both physiological and pathological conditions.

During ePG10 differentiation *zfh1* is specifically required for its survival

Several lines of evidence support the cell-autonomous action of *zfh1* in the survival of the subperineurial cell ePG10. First, in the PNS, *Zfh1* is specifically expressed by all embryonic PG. Second, in *zfh1* mutants, the death of ePG10 can be prevented by targeted glial-specific expression of *zfh1*. Third, over-expression of the viral caspase inhibitor p35 or the anti-apoptotic molecule *Diap1* in postmitotic glial cells in a *zfh1* mutant background prevents ePG10 cell fragmentation and apoptosis. Of note, rescues with *UAS-p35* and *UAS-Diap1* seem more efficient than with *UAS-zfh1*. One possible explanation is that p35 and *Diap1* interfere directly with the cell death machinery, whereas for *Zfh1* intermediate transcriptional events are necessary to block apoptosis. This interpretation would suggest that in ePG10 pro-apoptotic signals need to be held in check soon after its birth, that is around stage 12.

Of particular importance, in *zfh1* mutants in which ePG10 is rescued by p35, the molecular identity of this particular glial cell seems normal as it is Repo⁺, *Glialactin*⁺, *wingless*⁺, *mirror*⁺ and *Mz97*⁺ and its shape and migratory behaviour is comparable to wild type. These observations indicate that *zfh1* does not seem to have an instructive role for aspects of ePG10 differentiation other than survival, and that ePG10 cell death in *zfh1* mutant embryos is likely not a consequence of impaired differentiation.

It is notable that intriguing links between factors that promote cell migration and those that control cell survival have been reported recently, as both events appear often to be induced or inhibited by the same molecules (Montell, 2006). During a given differentiation process, the cell migration and cell survival processes seem tightly linked as cells 'live to migrate' and 'migrate to live' (Montell, 2006). Interestingly, a recent report shows that a precise temporal and quantitative regulation of Numb and Notch activities is required for ePG10 migration (Edenfeld *et al*, 2007) without affecting its survival. Our observations, together with the results of Edenfeld *et al* (2007) indicate that, at least for ePG10, survival and migration mechanisms can be molecularly dissociated.

Cell survival requirement within different subtypes of PG

Despite the fact that *Zfh1* is expressed by all PG, all three *zfh1* mutant alleles used in this study (*zfh1*⁵, *zfh1*^{75.26} and *zfh1*^{LacZ}) exhibit a highly penetrant phenotype only in ePG10. We cannot completely exclude, however, that other glial cells will be affected in *zfh1*⁵ or *zfh1*^{75.26} mutants at later stages, but due to the severe disorganization of several tissues and to their embryonic lethality we could not test this hypothesis. However, use of the hypomorphic *zfh1*^{LacZ} allele allowed us to determine that even at larval stage L1 only ePG10 seem to be affected whereas other glial cells develop

grossly normally. This observation underlines that ePG10 is more sensitive to loss of *zfh1* function and might reflect the singularity of this cell.

Indeed, within the PG born at the periphery, ePG10 is the only subtype able to migrate over long distances as a single cell (Umesono *et al*, 2002; Edenfeld *et al*, 2007). It is also known that glia form a heterogeneous cell population in terms of shape, structure or molecular identity (von Hilchen *et al*, 2008). Interestingly, regarding its physiological role, ePG10 belongs to the SPG subtype (*gliotactin*⁺). SPG form a thin layer below the perineurial cells, ensheath the peripheral nerve and insulate it against the potassium-rich hemolymph (Auld *et al*, 1995; Stork *et al*, 2008; von Hilchen *et al*, 2008). As suggested by electron microscopy analyses, only few SPG populate the peripheral nerve, they are very large and can form autocellular septate junctions (Stork *et al*, 2008). These observations are in agreement with earlier studies that have shown that during larval life the SPG do not divide but grow enormously large in size (Sepp *et al*, 2000; Schulte *et al*, 2003, 2006). As cell division or apoptosis of such cell type would imply disintegration of septate junctions and thus result in the opening of the blood-brain barrier, one can consider that SPG are under strict cell cycle and cell survival control. There may thus exist intrinsic differences between glial cell types that make them more or less sensitive to the balance between death and pro-survival signals. Our data indicate that ePG10 is highly susceptible to the balance between pro- and anti-apoptotic signals. First, we have noted using a hypomorphic allele of *Diap1* (*th^{ISC}*) that ePG10 is the only PG to undergo apoptosis during stages 13–16 in *th^{ISC}−/−* mutant embryos. Second, in *zfh1* mutant embryos carrying only one copy of the *H99* (or *XR38*) deficiency, ePG10 cell death is reverted. Third, ectopic activation of JNK pathway using *HepAct* in *zfh1*⁵ heterozygotes leads to ePG10 cell death whereas other PG appear unaffected.

***Zfh1* as a new transcriptional regulator influencing RHG-motifs genes expression**

It has been previously proposed that apoptosis may be controlled transcriptionally (Jacobson *et al*, 1997). The transcriptional regulation of the three classical cell death activators *reaper*, *hid* and *grim* has emerged as an important issue that has been addressed within different cellular systems in which apoptosis takes place. For example, during histolysis of larval salivary glands and midgut, signalling by steroid hormone activates *reaper* and *hid* expression (Jiang *et al*, 1997). In response to irradiation, the *Drosophila* p53 homologue has been shown to activate *reaper* (Brodsky *et al*, 2000). During development, the Hox gene *deformed* can directly activate *reaper* expression at the segmental boundaries in the maxillary segment (Lohmann *et al*, 2002).

Interestingly, in some other developing tissues such as the salivary gland, PCD does not occur (Myat and Andrew, 2000). In this model, it has been found that *senseless* (*sens*) together with *Fork head* (*fkh*) function as anti-apoptotic transcription factors by preventing the expression of *reaper* and *hid*. By blocking these pro-apoptotic genes, they allow survival of the salivary gland cells in the embryo (Myat and Andrew, 2000; Chandrasekaran and Beckendorf, 2003). In this study we have analysed SPG that are thought to be under strict cell cycle and survival control (Stork *et al*, 2008). By expression

analyses and genetics we have found that, among the four earlier well-characterized contiguous RHG-motif genes (*hid*, *grim*, *rpr* and *sickle*), *rpr* is the major cell death executor in ePG10 in *zfh1* mutants. At the expression level, using two different *rpr^{Gal4}* enhancer trap lines we have found that in *zfh1⁵* mutants, *rpr* is upregulated in this cell. Quantifications showed that this occurs in 10% of the hemisegments at stages 13–14. However, this percentage is likely to be an under-representation, and several arguments could account for such occasional *rpr* deregulation: (i) we have described that in *zfh1⁵* mutants, death of ePG10 is a progressive process spanning from late stage 12 up to stage 16 (see Figure 3H), which might reflect a dynamic *rpr* expression. Indeed, this has been already reported by Miguel-Aliaga and Thor (2004) within the embryonic nervous system and in naturally dying dMP2 neurons; (ii) the time required for *rpr^{Gal4}* levels to become high enough to be detected before the death of ePG10 may also represent an important limitation; (iii) finally, both *rpr^{Gal4}* insertions represent hypomorphic *rpr* alleles, and in our system, reducing *rpr* gene dosage leads to a significant reversion of ePG10 apoptosis (from 75 to 35%), which might reduce the probability of detecting *rpr* upregulation in a dying ePG10 cell in a given embryo at a given stage. At the genetic level, using combinations of alleles affecting *hid*, *grim*, *rpr* and/or *sickle*, we concluded that *rpr* is the major determinant of ePG10 apoptosis. Indeed, *zfh1* mutants carrying either a *H99* deletion (which removes *hid*, *grim* and *rpr*) or a *XR38* deletion (which removes *rpr* and *sickle*) or a hypomorphic *rpr* allele, ePG10 survive in a similar manner, that is in ~70% of hemisegments. Intriguingly, this number reaches 93 and 88% using *UAS-Diap1* or *UAS-p35*, respectively, raising the possibility that *Jafrac2*, the last known member of the RHG-motif gene family, could be implicated. As no loss of function allele has been so far reported for this gene, we were not able to test this hypothesis. Our results thus constitute a new example illustrating how, in cells devoid of PCD, the active transcriptional repression of RHG-motif genes is a defining factor enabling cell survival.

Fitting Zfh1 into the JNK signalling pathway for glial cell survival

In tissues normally devoid of PCD, what are the upstream pro-apoptotic signalling pathways that have to be actively held in check to ensure cell survival? How do these signalling interact with the transcriptional cascades involved in the regulation of RHG-motif genes expression?

In vertebrates, the JNK pathway regulates proliferation and/or death in several cell types (Leppa and Bohmann, 1999; Ham *et al*, 2000). Interestingly, in Schwann cells this pathway is required for TGF β -induced apoptosis (Parkinson *et al*, 2001). Our study also points for the first time towards a key role for the JNK signalling in the regulation of PG cell survival/death in *Drosophila*. Indeed, the JNK pathway is activated in ePG10 in *zfh1* mutants, and blocking this pathway (using Bsk^{DN}) reverts the *zfh1* phenotype (ePG10 cell death). These results indicate that Zfh1 is epistatic to JNK signalling. It is well established that JNK pathway can induce RHG-motif gene expression (*rpr*, *hid*) leading to apoptosis (reviewed in Varfolomeev and Ashkenazi, 2004). As DIAP1 has been shown to be a negative regulator of the JNK pathway, Rpr by sequestering DIAP1, enhances its own expression by a feed-forward loop (Kuranaga *et al*, 2002; Ryoo *et al*,

2004). In our system, in light of our observation that *pucE69* expression is increased in ePG10 in *zfh1* mutants, several hypotheses can be proposed: (i) Zfh1 could block transcription of one or more component(s) of the JNK signalling, thus maintaining the pro-apoptotic activity of this pathway in a low or inactive state. Our gain of function experiments using HepAct showed that Zfh1 does not seem to influence the core components of the cascade but rather acts upstream or at the level of Hep. It also strongly supports the view that Zfh1 is able to prevent apoptosis by blocking downstream pro-apoptotic effectors triggered by JNK signalling; and (ii) Zfh1 could act downstream of the core JNK cascade by solely repressing *rpr* independently of its control of the JNK pathway. Indeed, the fact that *rpr* can enhance its own expression by recruiting the JNK pathway (as mentioned above) could explain why in *zfh1* mutants we observe activation of this signalling. These two possibilities are non-exclusive as Zfh1 may be able to repress in parallel components of the JNK pathway and *rpr* (Figure 8E). The fact that Zfh1 orthologs have been mainly described as transcriptional repressors (Postigo and Dean, 2000) is consistent with these hypotheses. Further analyses are however needed to test whether these actions are direct or indirect and to delineate what are the precise target(s) of Zfh1 in this pathway. The genetic interaction of Zfh1 with the JNK pathway that we have reported in glial cell survival could be a more general feature in other processes and cell types. Indeed, during the course of this study we have noticed that in *zfh1* mutant embryos JNK signalling is dramatically induced in fat body cells (in which Zfh1 is normally expressed, data not shown) thereby triggering massive cell death in this tissue (data not shown). In addition, it has been recently hypothesized that Zfh1 could act through the JNK pathway during neuromuscular junction growth (Vogler and Urban, 2008). Interestingly, a recent analysis on the role of *Zfhx1b*, one of the two vertebrate homologues of *zfh1*, has showed such an interaction during hippocampus formation. In this model, *Zfhx1b* seems to have an anti-apoptotic function in neuronal cells by acting as an indirect activator of a JNK-dependant pro-survival pathway (Miquelajauregui *et al*, 2007), whereas in our system we have shown that the JNK pathway displays a pro-apoptotic activity. However, this discrepancy can be explained by the fact that the JNK pathway functions in a cell type and stimulus-dependent manner, as its components can have opposing roles in apoptosis (reviewed in Lin, 2003; Varfolomeev and Ashkenazi, 2004). In conclusion, these data raise the possibility that the interaction between Zfh1 and the JNK pathway has been conserved during evolution (eventually through distinct mechanisms depending on the cellular context) with cell survival being the final outcome.

Materials and methods

Drosophila stocks and P-Gal4 lines analysis

All genetic crosses were performed at 25°C unless otherwise specified. The following fly stocks were used in this study: *w¹¹¹⁸* was used as a wild-type stock; *UAS-myc-EGFP^F* (denoted *UAS-EGFP^F*) (Allan *et al*, 2003); *UAS-nls-myc-EGFP* (denoted *UAS-nlsEGFP*) (Callahan *et al*, 1998) (from D van Meyel); *Repo^{Gal4}* (Sepp *et al*, 2001) (from U Gaul); *J29^{LacZ}* enhancer trap insertion that reflects *gliotactin* expression (Auld *et al*, 1995) (from C Klämbt); *zfh1⁵* (Lai *et al*, 1993) (obtained from JC Lai); *zfh1^{75.26}* (Broihier *et al*, 1998) (from R Lehmann); *zfh1⁸⁶⁵* (Justice *et al*, 1995); *UAS-diap1* (Peterson *et al*, 2003) (from K McCall), *UAS-puc* (Martin-

Blanco *et al*, 1998) (from S Noselli). The following lines *th^{15C8}* (Hay *et al*, 1995); *H99* deficiency (White *et al*, 1994), *UAS-p35* (Hay *et al*, 1994), *UAS-bsk^{DN}* (Adachi-Yamada *et al*, 1999b) and *UAS-zfh1* were obtained from the Bloomington stock centre. Over-expression of each transgene *UAS-p35*, *UAS-Diap1*, *UAS-Bsk^{DN}*, *UAS-zfh1*, *UAS-Puc* with *Repo-Gal4* in a wild-type situation is shown in Supplementary Figure S2A–E. The *XR38* deficiency (Peterson *et al*, 2002), *wingless^{LacZ}*, *mirror^{LacZ}* and *Mz97^{Gal4}* (von Hilchen *et al*, 2008) were provided by K White, O Vef and G Technau. The *rpr^{Gal4}* lines (103634, NP: 520) and (112155, NP: 368) were generated and mapped by the NP Consortium (<http://flymap.lab.nig.ac.jp/~dclust/getdb.html>) are referred to as *rpr^{Gal4(520)}* and *rpr^{Gal4(368)}* respectively, in this study. For precise mapping of the *rpr^{Gal4}* lines genomic DNA PCR was carried out using a sense primer located in the 5' end of the P-Gal4 vector (5'-gtttggagag tagcgacac-3') and an antisens primer (5'-caagcgaaggatctgctgct-3') located in the 5' end of the *rpr* gene. All mutants lines were kept over *CyO,Act-GFP* or *TM3,Ser,Act-GFP* balancer chromosomes.

Immunostaining of embryos and western-blot analysis

Immunolabelling was carried out as described earlier (Thor *et al*, 1999). For first instar larval (stL1) dissection embryos were preselected during the time when their main dorsal tracheae begin to fill with air, which represents 18 h after egg laying (AEL) and allowed to develop for further 3 h. First instar larvae (21 h AEL) were dissected as described in Baines and Bate (1998). The following antibodies were used: α -22C10 (1:40), α -Elav (1:50), α -Repo 8D12 (1:80), α -c-Myc 9E10 (1:50), α -Repo 8D12 (1:80), α -Prospero MR1A (1:40), Fas2 1D4 (1:40) and α - β Gal 40-1a (1:10) (all from Developmental Studies Hybridoma Bank), rabbit α -Zfh1 (Van Doren *et al*, 2003) (1:5000), rabbit α -cleaved caspase-3 (Asp175, Cell Signaling; 1:200), rabbit α -Grim (Claveria *et al*, 1998) (1:1000), rabbit α -Hid (Bergmann *et al*, 2002) (1:500), rabbit α -Rpr (Freel *et al*, 2008) (1:1000 for western blot), mouse α - β tubulin (T5168 clone, Sigma; 1:10 000 for western blot), mouse α -GFP (A11120

clone, Molecular Probes, Eugene, OR; 1:250), rabbit α - β Gal (Cappel; 1:5000), before use, the polyclonal α - β Gal, α -GFP, α -Grim, α -Hid antibodies were pre-absorbed against early stage wild-type embryos. Secondary antibodies Alexafluor488 and Cy3 were used at 1:500 and 1:2000, respectively. Tissues were imaged on a Bio-Rad confocal microscope. Double-labelled images were false coloured using Photoshop for the benefit of colour-blind readers. For reaper western blot, preparations of proteins extracts were carried out as described in Freel *et al* (2008) and we used chemiluminescent HRP substrate for detection (Immobilon Western, from Millipore).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank the Developmental Studies Hybridoma Bank at the University of Iowa, the Bloomington Stock Center and the DGRC in Kyoto Institute of Technology for antibodies and fly stocks. We also thank R Lehmann, JC Lai, C Klämbt, U Gaul, J Peterson, K McCall, M Torres, H Steller, S Noselli, K White, O Vef, G Technau, C Freel and S Kornbluth for generously sharing fly lines and antibodies. We are grateful to CE Henderson for his critical comments during the preparation of this manuscript. This work was funded by Agence Nationale pour la Recherche (ANR05JCJC005901; AP, AG), INSERM and Association Française contre les Myopathies (AFM); AG was supported by AFM.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Adachi-Yamada T, Fujimura-Kamada K, Nishida Y, Matsumoto K (1999a) Distortion of proximodistal information causes JNK-dependent apoptosis in *Drosophila* wing. *Nature* **400**: 166–169
- Adachi-Yamada T, Nakamura M, Irie K, Tomoyasu Y, Sano Y, Mori E, Goto S, Ueno N, Nishida Y, Matsumoto K (1999b) p38 mitogen-activated protein kinase can be involved in transforming growth factor beta superfamily signal transduction in *Drosophila* wing morphogenesis. *Mol Cell Biol* **19**: 2322–2329
- Allan DW, St Pierre SE, Miguel-Aliaga I, Thor S (2003) Specification of neuroepithelial cell identity by the integration of retrograde BMP signaling and a combinatorial transcription factor code. *Cell* **113**: 73–86
- Auld VJ, Fetter RD, Broadie K, Goodman CS (1995) Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in *Drosophila*. *Cell* **81**: 757–767
- Baines RA, Bate M (1998) Electrophysiological development of central neurons in the *Drosophila* embryo. *J Neurosci* **18**: 4673–4683
- Bergmann A, Tugentman M, Shilo BZ, Steller H (2002) Regulation of cell number by MAPK-dependent control of apoptosis: a mechanism for trophic survival signaling. *Dev Cell* **2**: 159–170
- Borderie VM, Baudrimont M, Vallee A, Ereau TL, Gray F, Laroche L (2000) Corneal endothelial cell apoptosis in patients with Fuchs' dystrophy. *Invest Ophthalmol Vis Sci* **41**: 2501–2505
- Brennecke J, Hipfner DR, Stark A, Russell RB, Cohen SM (2003) bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene *hid* in *Drosophila*. *Cell* **113**: 25–36
- Brodsky MH, Nordstrom W, Tsang G, Kwan E, Rubin GM, Abrams JM (2000) *Drosophila* p53 binds a damage response element at the reaper locus. *Cell* **101**: 103–113
- Broihier HT, Moore LA, Van Doren M, Newman S, Lehmann R (1998) *zfh-1* is required for germ cell migration and gonadal mesoderm development in *Drosophila*. *Development* **125**: 655–666
- Bui T, Sequeira J, Wen TC, Sola A, Higashi Y, Kondoh H, Genetta T (2009) ZEB1 links p63 and p73 in a novel neuronal survival pathway rapidly induced in response to cortical ischemia. *PLoS ONE* **4**: e4373
- Callahan CA, Yoshikawa S, Thomas JB (1998) Tracing axons. *Curr Opin Neurobiol* **8**: 582–586
- Campbell G, Goring H, Lin T, Spana E, Andersson S, Doe CQ, Tomlinson A (1994) RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in *Drosophila*. *Development* **120**: 2957–2966
- Chandrasekaran V, Beckendorf SK (2003) senseless is necessary for the survival of embryonic salivary glands in *Drosophila*. *Development* **130**: 4719–4728
- Chen P, Nordstrom W, Gish B, Abrams JM (1996) grim, a novel cell death gene in *Drosophila*. *Genes Dev* **10**: 1773–1782
- Claveria C, Albar JP, Serrano A, Buesa JM, Barbero JL, Martinez AC, Torres M (1998) *Drosophila* grim induces apoptosis in mammalian cells. *EMBO J* **17**: 7199–7208
- Edenfeld G, Altenhein B, Zierau A, Cleppien D, Krukkert K, Technau G, Klämbt C (2007) Notch and Numb are required for normal migration of peripheral glia in *Drosophila*. *Dev Biol* **301**: 27–37
- Fortini ME, Lai ZC, Rubin GM (1991) The *Drosophila* *zfh-1* and *zfh-2* genes encode novel proteins containing both zinc-finger and homeodomain motifs. *Mech Dev* **34**: 113–122
- Freel CD, Richardson DA, Thomenius MJ, Gan EC, Horn SR, Olson MR, Kornbluth S (2008) Mitochondrial localization of Reaper to promote inhibitors of apoptosis protein degradation conferred by GH3 domain-lipid interactions. *J Biol Chem* **283**: 367–379
- Galko MJ, Krasnow MA (2004) Cellular and genetic analysis of wound healing in *Drosophila* larvae. *PLoS Biol* **2**: E239
- Garces A, Thor S (2006) Specification of *Drosophila* aCC motoneuron identity by a genetic cascade involving even-skipped, grain and *zfh1*. *Development* **133**: 1445–1455
- Goyal L, McCall K, Agapite J, Hartwig E, Steller H (2000) Induction of apoptosis by *Drosophila* reaper, hid and grim through inhibition of IAP function. *EMBO J* **19**: 589–597
- Grether ME, Abrams JM, Agapite J, White K, Steller H (1995) The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. *Genes Dev* **9**: 1694–1708

- Halter DA, Urban J, Rickert C, Ner SS, Ito K, Travers AA, Technau GM (1995) The homeobox gene *repo* is required for the differentiation and maintenance of glia function in the embryonic nervous system of *Drosophila melanogaster*. *Development* **121**: 317–332
- Ham J, Eilers A, Whitfield J, Neame SJ, Shah B (2000) c-Jun and the transcriptional control of neuronal apoptosis. *Biochem Pharmacol* **60**: 1015–1021
- Hay BA, Wassarman DA, Rubin GM (1995) *Drosophila* homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**: 1253–1262
- Hay BA, Wolff T, Rubin GM (1994) Expression of baculovirus P35 prevents cell death in *Drosophila*. *Development* **120**: 2121–2129
- Igaki T, Kanda H, Yamamoto-Goto Y, Kanuka H, Kuranaga E, Aigaki T, Miura M (2002) Eiger, a TNF superfamily ligand that triggers the *Drosophila* JNK pathway. *EMBO J* **21**: 3009–3018
- Jacobson MD, Weil M, Raff MC (1997) Programmed cell death in animal development. *Cell* **88**: 347–354
- Jiang C, Baehrecke EH, Thummel CS (1997) Steroid regulated programmed cell death during *Drosophila* metamorphosis. *Development* **124**: 4673–4683
- Justice RW, Zilian O, Woods DF, Noll M, Bryant PJ (1995) The *Drosophila* tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev* **9**: 534–546
- Krafchak CM, Pawar H, Moroi SE, Sugar A, Lichter PR, Mackey DA, Mian S, Nairus T, Elner V, Schteingart MT, Downs CA, Kijek TG, Johnson JM, Trager EH, Rozsa FW, Mandal MN, Epstein MP, Vollrath D, Ayyagari R, Boehnke M *et al* (2005) Mutations in TCF8 cause posterior polymorphous corneal dystrophy and ectopic expression of COL4A3 by corneal endothelial cells. *Am J Hum Genet* **77**: 694–708
- Kuranaga E, Kanuka H, Igaki T, Sawamoto K, Ichijo H, Okano H, Miura M (2002) Reaper-mediated inhibition of DIAP1-induced DTRAF1 degradation results in activation of JNK in *Drosophila*. *Nat Cell Biol* **4**: 705–710
- Lai ZC, Rushton E, Bate M, Rubin GM (1993) Loss of function of the *Drosophila* *zfh-1* gene results in abnormal development of mesodermally derived tissues. *Proc Natl Acad Sci USA* **90**: 4122–4126
- Layden MJ, Odden JP, Schmid A, Garces A, Thor S, Doe CQ (2006) Zfh1, a somatic motor neuron transcription factor, regulates axon exit from the CNS. *Dev Biol* **291**: 253–263
- Lee HK, Lundell MJ (2007) Differentiation of the *Drosophila* serotonergic lineage depends on the regulation of Zfh-1 by Notch and Eagle. *Mol Cell Neurosci* **36**: 47–58
- Leppa S, Bohmann D (1999) Diverse functions of JNK signaling and c-Jun in stress response and apoptosis. *Oncogene* **18**: 6158–6162
- Li QJ, Ashraf MF, Shen DF, Green WR, Stark WJ, Chan CC, O'Brien TP (2001) The role of apoptosis in the pathogenesis of Fuchs endothelial dystrophy of the cornea. *Arch Ophthalmol* **119**: 1597–1604
- Lin A (2003) Activation of the JNK signaling pathway: breaking the brake on apoptosis. *Bioessays* **25**: 17–24
- Lohmann I, McGinnis N, Bodmer M, McGinnis W (2002) The *Drosophila* Hox gene *deformed* sculpts head morphology via direct regulation of the apoptosis activator reaper. *Cell* **110**: 457–466
- Martin-Blanco E, Gampel A, Ring J, Virdee K, Kirov N, Tolkovsky AM, Martinez-Arias A (1998) *puckered* encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev* **12**: 557–570
- McEwen DG, Peifer M (2005) *Puckered*, a *Drosophila* MAPK phosphatase, ensures cell viability by antagonizing JNK-induced apoptosis. *Development* **132**: 3935–3946
- Meier P, Silke J, Leever SJ, Evan GI (2000) The *Drosophila* caspase DRONC is regulated by DIAP1. *EMBO J* **19**: 598–611
- Miguel-Aliaga I, Thor S (2004) Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. *Development* **131**: 6093–6105
- Miquelajauregui A, Van de Putte T, Polyakov A, Nityanandam A, Boppana S, Seuntjens E, Karabinos A, Higashi Y, Huylebroeck D, Tarabkin V (2007) Smad-interacting protein-1 (Zfhx1b) acts upstream of Wnt signaling in the mouse hippocampus and controls its formation. *Proc Natl Acad Sci USA* **104**: 12919–12924
- Montell DJ (2006) The social lives of migrating cells in *Drosophila*. *Curr Opin Genet Dev* **16**: 374–383
- Moore LA, Broihier HT, Van Doren M, Lehmann R (1998) Gonadal mesoderm and fat body initially follow a common developmental path in *Drosophila*. *Development* **125**: 837–844
- Mowat DR, Wilson MJ, Goossens M (2003) Mowat-Wilson syndrome. *J Med Genet* **40**: 305–310
- Muro I, Hay BA, Clem RJ (2002) The *Drosophila* DIAP1 protein is required to prevent accumulation of a continuously generated, processed form of the apical caspase DRONC. *J Biol Chem* **277**: 49644–49650
- Myat MM, Andrew DJ (2000) Fork head prevents apoptosis and promotes cell shape change during formation of the *Drosophila* salivary glands. *Development* **127**: 4217–4226
- O'Neill EM, Rebay I, Tjian R, Rubin GM (1994) The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* **78**: 137–147
- Parkinson DB, Dong Z, Bunting H, Whitfield J, Meier C, Marie H, Mirsky R, Jessen KR (2001) Transforming growth factor beta (TGFbeta) mediates Schwann cell death *in vitro* and *in vivo*: examination of c-Jun activation, interactions with survival signals, and the relationship of TGFbeta-mediated death to Schwann cell differentiation. *J Neurosci* **21**: 8572–8585
- Peterson C, Carney GE, Taylor BJ, White K (2002) reaper is required for neuroblast apoptosis during *Drosophila* development. *Development* **129**: 1467–1476
- Peterson JS, Barkett M, McCall K (2003) Stage-specific regulation of caspase activity in *drosophila* oogenesis. *Dev Biol* **260**: 113–123
- Postigo AA, Dean DC (2000) Differential expression and function of members of the *zfh-1* family of zinc finger/homeodomain repressors. *Proc Natl Acad Sci USA* **97**: 6391–6396
- Ramet M, Lanot R, Zachary D, Manfrulli P (2002) JNK signaling pathway is required for efficient wound healing in *Drosophila*. *Dev Biol* **241**: 145–156
- Ryoo HD, Gorenc T, Steller H (2004) Apoptotic cells can induce compensatory cell proliferation through the JNK and the Wingless signaling pathways. *Dev Cell* **7**: 491–501
- Schulte J, Charish K, Que J, Ravn S, MacKinnon C, Auld VJ (2006) Gliotactin and Discs large form a protein complex at the tricellular junction of polarized epithelial cells in *Drosophila*. *J Cell Sci* **119**: 4391–4401
- Schulte J, Tepass U, Auld VJ (2003) Gliotactin, a novel marker of tricellular junctions, is necessary for septate junction development in *Drosophila*. *J Cell Biol* **161**: 991–1000
- Sepp KJ, Auld VJ (2003) Reciprocal interactions between neurons and glia are required for *Drosophila* peripheral nervous system development. *J Neurosci* **23**: 8221–8230
- Sepp KJ, Schulte J, Auld VJ (2000) Developmental dynamics of peripheral glia in *Drosophila melanogaster*. *Glia* **30**: 122–133
- Sepp KJ, Schulte J, Auld VJ (2001) Peripheral glia direct axon guidance across the CNS/PNS transition zone. *Dev Biol* **238**: 47–63
- Stork T, Engelen D, Krudewig A, Silies M, Bainton RJ, Klamt C (2008) Organization and function of the blood-brain barrier in *Drosophila*. *J Neurosci* **28**: 587–597
- Su MT, Fujioka M, Goto T, Bodmer R (1999) The *Drosophila* homeobox genes *zfh-1* and *even-skipped* are required for cardiac-specific differentiation of a numb-dependent lineage decision. *Development* **126**: 3241–3251
- Suzanne M, Perrimon N, Noselli S (2001) The *Drosophila* JNK pathway controls the morphogenesis of the egg dorsal appendages and micropyle. *Dev Biol* **237**: 282–294
- Tateno M, Nishida Y, Adachi-Yamada T (2000) Regulation of JNK by Src during *Drosophila* development. *Science* **287**: 324–327
- Thor S, Andersson SG, Tomlinson A, Thomas JB (1999) A LIM-homeodomain combinatorial code for motor-neuron pathway selection. *Nature* **397**: 76–80
- Umesono Y, Hiromi Y, Hotta Y (2002) Context-dependent utilization of Notch activity in *Drosophila* glial determination. *Development* **129**: 2391–2399
- Van Doren M, Mathews WR, Samuels M, Moore LA, Broihier HT, Lehmann R (2003) *fear of intimacy* encodes a novel transmembrane protein required for gonad morphogenesis in *Drosophila*. *Development* **130**: 2355–2364
- Varfolomeev EE, Ashkenazi A (2004) Tumor necrosis factor: an apoptosis *Jun*Kie? *Cell* **116**: 491–497
- Vogler G, Urban J (2008) The transcription factor Zfh1 is involved in the regulation of neuropeptide expression and growth of larval

- neuromuscular junctions in *Drosophila melanogaster*. *Dev Biol* **319**: 78–85
- von Hilchen CM, Beckervordersandforth RM, Rickert C, Technau GM, Altenhein B (2008) Identity, origin, and migration of peripheral glial cells in the *Drosophila* embryo. *Mech Dev* **125**: 337–352
- Wang SL, Hawkins CJ, Yoo SJ, Muller HA, Hay BA (1999) The *Drosophila* caspase inhibitor DIAP1 is essential for cell survival and is negatively regulated by HID. *Cell* **98**: 453–463
- White K, Grether ME, Abrams JM, Young L, Farrell K, Steller H (1994) Genetic control of programmed cell death in *Drosophila*. *Science* **264**: 677–683
- Wilson R, Goyal L, Ditzel M, Zachariou A, Baker DA, Agapite J, Steller H, Meier P (2002) The DIAP1 RING finger mediates ubiquitination of Dronc and is indispensable for regulating apoptosis. *Nat Cell Biol* **4**: 445–450
- Xiong WC, Okano H, Patel NH, Blendy JA, Montell C (1994) repo encodes a glial-specific homeo domain protein required in the *Drosophila* nervous system. *Genes Dev* **8**: 981–994
- Yu SY, Yoo SJ, Yang L, Zapata C, Srinivasan A, Hay BA, Baker NE (2002) A pathway of signals regulating effector and initiator caspases in the developing *Drosophila* eye. *Development* **129**: 3269–3278
- Zeitlinger J, Bohmann D (1999) Thorax closure in *Drosophila*: involvement of Fos and the JNK pathway. *Development* **126**: 3947–3956