

# Regulation of Cell Number by MAPK-Dependent Control of Apoptosis: A Mechanism for Trophic Survival Signaling

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## Summary

Trophic mechanisms in which neighboring cells mutually control their survival by secreting extracellular factors play an important role in determining cell number. However, how trophic signaling suppresses cell death is still poorly understood. We now show that the survival of a subset of midline glia cells in *Drosophila* depends upon direct suppression of the proapoptotic protein HID via the EGF receptor/RAS/MAPK pathway. The TGF $\alpha$ -like ligand SPITZ is activated in the neurons, and glial cells compete for limited amounts of secreted SPITZ to survive. In midline glia that fail to activate the EGFR pathway, HID induces apoptosis by blocking a caspase inhibitor, Diap1. Therefore, a direct pathway linking a specific extracellular survival factor with a caspase-based death program has been established.

## Introduction

The development of multicellular organisms depends on the recruitment of a large number of different cell types into tissues and organs. A tight balance between cell proliferation, cell differentiation, and cell death determines the control of cell fate and number. The trophic theory of cell survival provides a conceptual framework for understanding how programmed cell death (PCD) or apoptosis is dynamically regulated in the context of a developing organism. Originally developed to explain the massive neuronal cell loss during vertebrate CNS development, the trophic theory is based on the assumption that a cell-intrinsic suicide program operates by default unless it is suppressed by trophic (survival) factors secreted by neighboring cells (reviewed in Openheim, 1991; Raff, 1992; Raff et al., 1993). Since trophic factors are produced in limited amounts, the trophic mechanism ensures that only an appropriate number of neurons survive. Neurons that fail to project to the

proper targets do not receive sufficient trophic factors and are subsequently eliminated by PCD. This “social control” (Raff, 1992) of cell survival ensures the functional integrity of a given tissue or organ by matching the number of different cell types to each other. Importantly, it also implies that there are physiological mechanisms that prevent cells from dying by interfering with the cell death program.

The dependence on trophic signals from other cells for survival is not confined to neurons. The survival of many cell types depends on trophic mechanisms. Survival of cultured oligodendrocytes, an ensheathing-type glia in the vertebrate nervous system, can be promoted by factors produced by their neighboring astrocytes or by factors present in the optic nerve (Barres et al., 1993; Raff et al., 1993; Louis et al., 1993; Doyle and Colman, 1993). In addition, there is evidence that glial-axonal contact plays a role in the survival of glial cells in both *Drosophila* and mammals (Jacobs, 2000; Raff et al., 1993). However, the molecular mechanism by which axons promote glia survival is not clear.

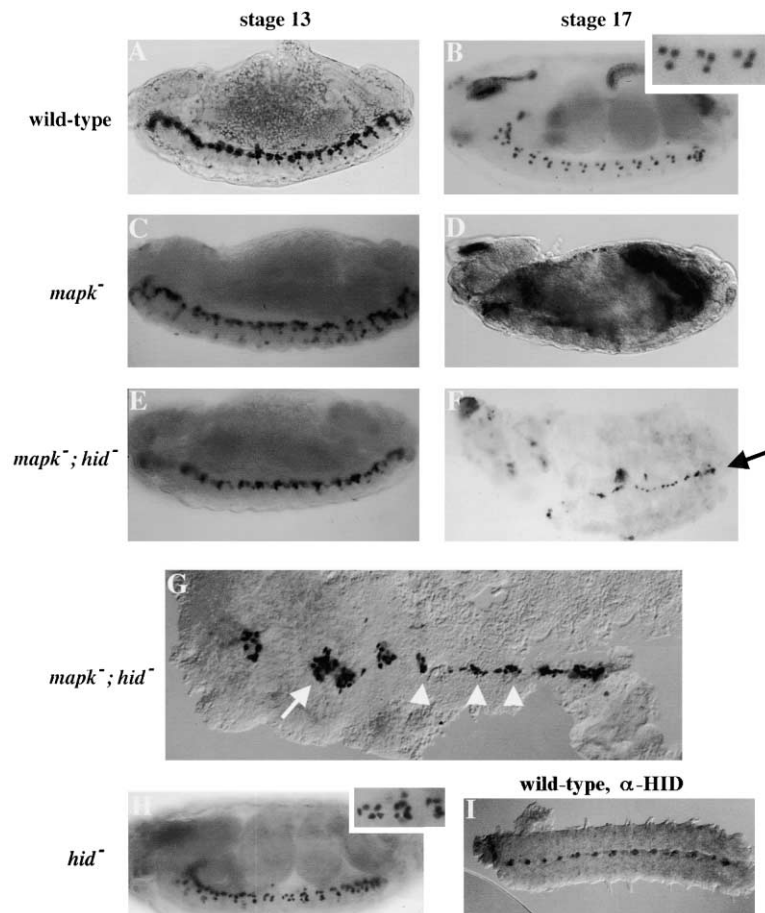
In *Drosophila*, large numbers of cells die during development (Abrams et al., 1993). As in vertebrates, the regulation of PCD in *Drosophila* is dependent on environmental circumstances and appears to involve trophic interactions. The basic cell death machinery is conserved in flies. Homologs of caspases, ced-4/APAF-1, ced-9/Bcl-2, and IAPs (inhibitor of apoptosis proteins) have been described in *Drosophila* (for a review, see Song and Steller, 1999). In addition, three novel cell death regulators, *reaper*, *head involution defective (hid)*, and *grim*, have been identified (White et al., 1994; Grether et al., 1995; Chen et al., 1996). These genes play a key role in integrating different signaling pathways that regulate the decision between cell death and cell survival (Song and Steller, 1999). Once activated, REAPER, HID, and GRIM kill by inhibiting the antiapoptotic function of an inhibitor of apoptosis protein (IAP), *diap1* (Goyal et al., 2000; Wang et al., 1999). Furthermore, expression of these proteins in mammalian cells and a *Xenopus* cell-free system is sufficient to induce apoptosis (Evans et al., 1997; Haining et al., 1999; McCarthy and Dixit, 1998). Recently, it has been suggested that Smac/Diablo represents a mammalian functional homolog of *reaper*, *hid*, and *grim* (Chai et al., 2000). Therefore, it is likely that findings obtained in studies of PCD in *Drosophila* will have broader implications for understanding the regulation of PCD in higher vertebrates, including mammals and humans.

The *reaper* and *grim* genes are transcriptionally induced in many cells which are doomed to die. Their expression pattern largely mimics the pattern of apoptosis in the *Drosophila* embryo (White et al., 1994; Chen et al., 1996). In contrast, the gene *hid* is expressed in both dying cells as well as in cells that live (Grether et al., 1995; this study). Since HID is a strong inducer of PCD, there must be posttranslational regulation of *hid* to suppress the death of surviving *hid*-expressing cells. One mechanism by which extracellular survival factors appear to suppress HID-induced apoptosis is through

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**Figure 1. MAPK-Mediated Survival of the MG Is through Inhibition of HID**

In this and the following figures (except Figure 6), the MG are detected using the *sli-lacZ* reporter transgene and  $\beta$ -gal immunohistochemistry.

(A–F) In wild-type embryos, a group of approximately ten *sli-lacZ*-expressing MG per segment is visible at stage 13 (A). During subsequent developmental stages, the majority of MG undergo apoptosis such that only about three MG survive by stage 17 (B). The insert in (B) shows the surviving MG in three segments. In *mapk* mutant embryos, the MG initially form (C), but fail to survive at later stages (D). Continued MG survival requires suppression of HID by MAPK as evidenced by *mapk*; *hid* double mutant analysis. In *mapk*; *hid* double mutant embryos, the MG form normally (E), and survive to the end of embryogenesis (F, black arrow) despite the fact that MAPK activity is missing.

(G) Dissection of the double mutant embryos reveals that the CNS does not develop very well and that the MG locate at the cuticle, unable to die. Due to segmental fusions in these embryos, we see groups of up to 20 MG (see arrow in [G]). In other segments, about five to six MG are detectable (see arrowheads).

(H) *hid* single mutant embryos contain a similar number of surviving MG, on average 5.8 per segment.

(I) The nerve cord of a late stage wild-type embryo stained with anti-HID antibody. The only cells expressing HID as this stage are MG.

activation of the RAS/MAPK pathway (Sawamoto et al., 1998; Kurada and White, 1998; Bergmann et al., 1998). The MAPK phosphorylation sites in HID are critical for the antiapoptotic effect of RAS/MAPK signaling, suggesting that phosphorylation by MAPK inactivates HID (Bergmann et al., 1998). However, while the mechanisms for HID inactivation have been characterized, their capacity to shape a tissue under a normal setting, rather than ectopic expression, has not yet been demonstrated.

For this purpose, we have examined the cell death of the embryonic midline glia (MG) during the formation of the *Drosophila* central nervous system (Klambt et al., 1991; for a review, see Jacobs, 2000). The MG are required for the separation of commissural axon tracts and their ensheathment (Klambt et al., 1991). Initially (at stage 13 of embryogenesis), about ten MG cells per segment are generated. During the following stages, most of the MG undergo apoptosis, leaving typically three ensheathing MG per segment to survive after axonal connections have been established (at stage 17; Klambt et al., 1991; Sonnenfeld and Jacobs, 1995; Zhou et al., 1995). The death of MG cells appears to be stochastic and correlates with glial-axonal interaction: if axonal contact is disrupted, the MG undergo apoptosis (Sonnenfeld and Jacobs, 1995; Noordermeer et al., 1999). Furthermore, MG apoptosis requires the activities of *reaper*, *hid*, and *grim* (Dong and Jacobs, 1997; Sonnenfeld and Jacobs, 1995; Zhou et al., 1995, 1997). Interestingly, *hid* is required for the cell death of about half the

MG, and *reaper* and *grim* appear to control apoptosis in the remaining subset of the MG (Zhou et al., 1997).

*Drosophila* EGF receptor (EGFR) signaling is important for the differentiation and survival of MG cells (Sonnenfeld and Jacobs, 1994; Stemerding and Jacobs, 1997; Scholz et al., 1997; see also Yamada et al., 1997). EGFR signaling has also been implicated in differentiation and survival of photoreceptor cells in the developing retina (Baker and Yu, 2001). Here, we examine the regulation of MG survival by the EGFR pathway. We find that the survival of a subset of MG cells depends on the inactivation of HID through phosphorylation by MAPK, and that this process in turn requires the activation of the EGFR by one of its ligands, SPITZ. MG survival requires cell-cell contact between MG cells and axons which triggers processing and secretion of active SPITZ by the axons. We conclude that SPITZ functions as a survival factor for the MG by specifically antagonizing the proapoptotic activity of HID via activation of the EGFR/RAS/MAPK pathway. These findings provide a molecular model for how trophic signals block the intrinsic cell death machinery in general, and how axons promote the survival of *Drosophila* midline glia cells in particular.

## Results

The reduction in midline glia (MG) cell number due to apoptosis, as well as the requirement of the RAS/MAPK

pathway for MG survival, has been previously documented, using various MG-specific enhancer trap lines and reporter fusion constructs (Sonnenfeld and Jacobs, 1995; Zhou et al., 1995, 1997; Scholz et al., 1997; Dong and Jacobs, 1997; Stemerink and Jacobs, 1997). In this paper, we visualize the MG using a reporter fusion construct for the *slit* gene (*slit-lacZ*) in which a 1 kb fragment of the *slit* promoter confers expression specifically to the MG (Wharton and Crews, 1993; Sonnenfeld and Jacobs, 1995). Using a  $\beta$ -gal antibody to monitor the developmental profile of the MG, about ten cells per segment expressing *slit-lacZ* are detectable at midembryogenesis (stage 13; see Figure 1A; Zhou et al., 1997). By the end of embryogenesis at stage 17, the number of *slit-lacZ*-positive cells is reduced to approximately three per segment (on average, 2.8;  $n = 189$ ; see Figure 1B). Since the *slit-lacZ* expression is specific for the MG, we refer to *slit-lacZ*-expressing cells from now on as MG.

#### MAPK-Mediated Survival of the MG Is through Inhibition of HID

Prominent activation of MAPK was identified in the MG cells (Gabay et al., 1997a, 1997b), but its functional role has not been determined. We analyzed the fate of the MG in *mapk*-deficient embryos. Compared to wild-type embryos, the initial generation of the MG appears to be normal (compare the stage 13 embryos in Figures 1A and 1C). However, by stage 17 (the end of embryogenesis), none of the MG in *mapk*-deficient embryos survived (Figure 1D). This finding suggests that MAPK is required for MG survival in wild-type embryos (Figure 1B).

The genetic requirement of *mapk* for MG survival and of *hid* for MG apoptosis (see Introduction; Figure 2C; Zhou et al., 1997) prompted us to assume that MAPK promotes survival of the MG by inhibition of HID activity. According to this model, the MG would be unprotected from HID-induced apoptosis in *mapk*-deficient embryos, and die. Consistent with this idea, HID protein is detectable in the MG of late stage wild-type embryos (Figure 1I). To test this further, we analyzed embryos which are mutant for both *mapk* and *hid*. In early stage *mapk; hid* double mutant embryos, the initial generation of the MG appears to be normal (Figure 1E). However, in contrast to *mapk* mutants alone, the MG is rescued in *mapk; hid* double mutant embryos although the survival function of MAPK is missing in these embryos (Figure 1F). Dissection revealed that the MG are located directly at the cuticle of the embryos (Figure 1G). Because segmental fusions occur in these embryos, some of the MG cluster in groups of up to 20 cells (see arrow in Figure 1G). In individual segments, five to six MG are visible (see arrowheads in Figure 1G). This number is larger compared to wild-type (three MG per segment; Figure 1B), and is remarkably similar to the number of surviving MG in *hid* mutant embryos alone (Figure 1H; see next section), indicating that MAPK promotes MG survival largely through inhibition of HID.

The mutant analysis revealed that MAPK is required to suppress the activity of HID for MG survival. If *hid* is mutant in *mapk*-deficient embryos (i.e., in *mapk; hid* double mutants), MAPK is no longer needed for the survival of the MG. Thus, MAPK-mediated survival of the MG functions through inhibition of HID.

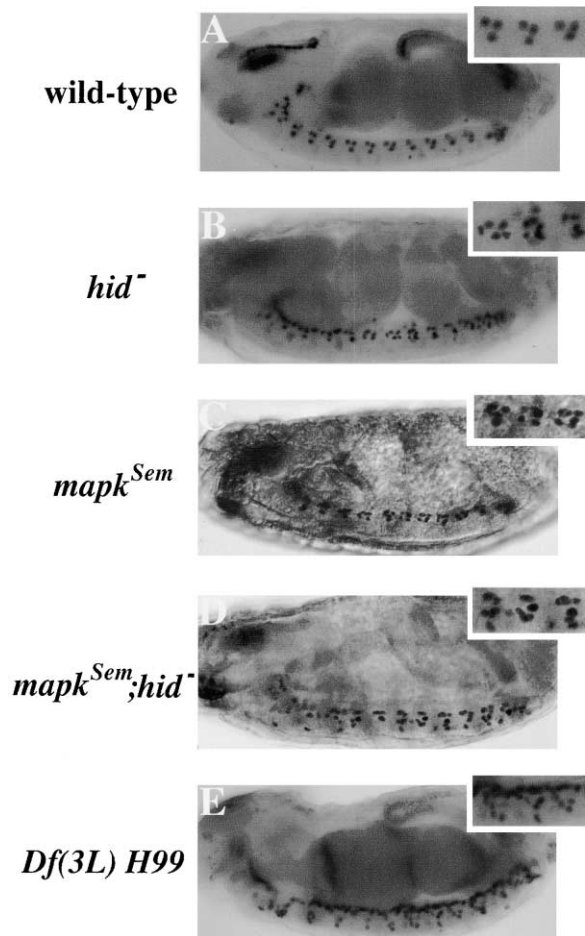
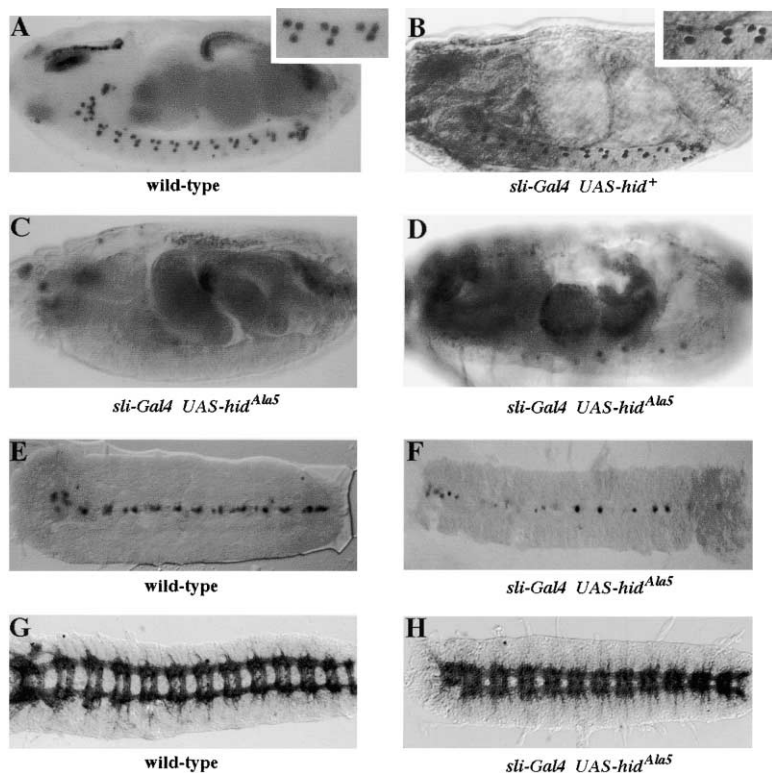


Figure 2. HID and MAPK Coordinate MG Cell Number

In *hid* mutant embryos (B), the number of surviving MG per segment is increased compared to wild-type (A) from 2.8 to 5.8. Mutational activation of MAPK by the mutant allele *mapk<sup>Sem</sup>* results in survival of 6.0 MG (C), a number very similar to the one obtained in *hid* embryos (B). Double mutant analysis with *mapk<sup>Sem</sup>* and *hid* reveals on average 6.6 surviving MG (D), indicating that activation of *mapk<sup>Sem</sup>* and expression of *hid* occur in largely the same MG. For comparison, if MG survival in *hid* and *mapk<sup>Sem</sup>* were independent of each other, then the double mutant should reveal a similar number of surviving MG as seen in *Df(3L)H99* embryos (E), that is, about ten to twelve MG per segment. The inserts in all panels show enlarged views of three segments each. The *hid* allele used is *hid<sup>WR+X1</sup>*.

#### MAPK Activity Level Determines the Extent of MG Survival

Zhou et al. (1997) noted that in *hid* mutant embryos there is a 2-fold increase of the MG compared to wild-type. Approximately six MG per segment (on average, 5.8;  $n = 182$ ) survive in *hid* embryos compared to the 2.8 MG per segment in wild-type (Figures 2A and 2B), indicating that *hid* is genetically required for MG cell death. We have shown above that MAPK activity is required for MG survival. Does the level of MAPK activity determine the final number of surviving MG cells? Mutational activation of MAPK, using a dominant allele of MAPK termed *Sevenmaker* or *mapk<sup>Sem</sup>*, promotes survival of extra MG. About 6.0 MG per segment ( $n = 231$ ) survive in stage 17 *mapk<sup>Sem</sup>* embryos (Figure 2C), providing additional



**Figure 3. The MAPK-Unresponsive  $HID^{Ala5}$  Mutant Induces Apoptosis in the MG**

Using the UAS/GAL4 expression system, it was shown that expression of  $hid^{+}$  under control of the MG-specific *sli-GAL4* driver failed to induce apoptosis in the MG (compare the wild-type embryo in [A] with the *sli-GAL4/UAS-hid<sup>+</sup>* embryo in [B]; Zhou et al., 1997). This failure is due to MAPK-dependent inactivation of additional HID protein. *UAS-hid<sup>Ala5</sup>* transgenes, in which the five MAPK phosphorylation sites of HID are changed to Ala residues, cause either complete (C) or, due to reduced expression levels, partial (D) MG ablation. Nerve cord preparation of the embryo in (D) reveals that in this embryo, only a fraction of the wild-type complement of the MG survives (compare [E] and [F]). The inserts in (A) and (B) show enlarged views of three segments each.

The MG are required to separate anterior from posterior commissure axon tracts (see [G] for a wild-type embryo, which has been stained with the antibody BP102 to label the CNS axon pattern). However, expression of  $HID^{Ala5}$  in the MG causes a fused commissure phenotype (H), indicating loss of MG activity.

evidence that MAPK is required for MG survival. Remarkably, the number of surviving MG in *mapk<sup>Sem</sup>* embryos is very similar to the number of surviving MG in *hid* mutant embryos. In both cases, approximately six MG survive per segment (compare Figures 2B and 2C). Therefore, we determined whether the six surviving MG in *mapk<sup>Sem</sup>* embryos correspond to the same MG that survive in *hid* mutant embryos by double mutant analysis. Stage 17 *mapk<sup>Sem</sup>; hid* double mutant embryos contain on average 6.6 MG ( $n = 91$ ) per segment (Figure 2D), or slightly more than the single mutants alone. This result strongly suggests that *hid* expression and MAPK activation occur in largely the same set of MG, that is, in a group of about six MG. If MAPK activation and *hid* expression would occur in different MG independently of each other, then the *mapk<sup>Sem</sup>; hid* double mutant would be expected to be the composite of the individual mutants and a total of about ten to twelve MG would survive in the double mutant, similar to what has been observed in *H99* mutant embryos (Figure 2E; Zhou et al., 1995; Sonnenfeld and Jacobs, 1995). We infer from the double mutant analysis (Figure 2D) that the survival of approximately six MG is regulated by MAPK-dependent inhibition of HID. As long as MAPK is activated, these MG survive (as seen in the activated *mapk<sup>Sem</sup>* mutant). However, MG in this group that does not maintain activated MAPK are eliminated by HID-induced apoptosis. Thus, the coordinated expression of HID and activation of MAPK regulate the final MG cell number (see also Discussion).

#### MG Survival Requires Phosphorylation of HID by MAPK

MAPK suppresses *hid* activity in two ways: via downregulation of its transcription and via phosphorylation of

HID protein (Kurada and White, 1998; Bergmann et al., 1998). However, *hid* mRNA and protein are readily detectable in the surviving MG of wild-type embryos (Figure 1I; Zhou et al., 1997). Therefore, transcriptional downregulation of *hid* does not account for MG survival. This prompted us to test whether inhibitory phosphorylation of HID by MAPK might be critical for MG survival. For this purpose, we took advantage of an observation by Zhou et al. (1997). Overexpression of HID in the MG using the MG-specific *sli-GAL4* driver and *UAS-hid* transgenes is not sufficient to induce MG apoptosis (Figure 3B). Even two copies of the *UAS-hid* transgenes were not able to ablate the MG (Zhou et al., 1997). This is contrary to findings in other tissues in which expression of *hid* induces cell death very well (Grether et al., 1995; Bergmann et al., 1998). However, since MAPK is activated in the MG (Gabay et al., 1997a, 1997b) and required for MG survival (this study), we hypothesized that even overexpressed HID might be inactivated via MAPK phosphorylation.

To examine this further, we generated *UAS-hid* transgenes that alter the five phosphoacceptor residues of the MAPK phosphorylation sites to nonphosphorylatable Ala residues (*UAS-hid<sup>Ala5</sup>*; Bergmann et al., 1998). The *UAS-hid<sup>Ala5</sup>* transgenes driven by *sli-GAL4* induce apoptosis in the MG very efficiently. One copy of a *UAS-hid<sup>Ala5</sup>* transgene is sufficient for the ablation of the MG. Occasionally, some embryos are recovered in which the ablation of the MG is incomplete (Figure 3D). However, nerve cord preparations reveal that in these embryos, only a small fraction of the MG survives compared to wild-type (Figures 3E and 3F). Some segments completely lack MG cells, while others just contain one remaining MG (Figure 3F). The MG is required for separation of the commissural axon tracts of the CNS (see

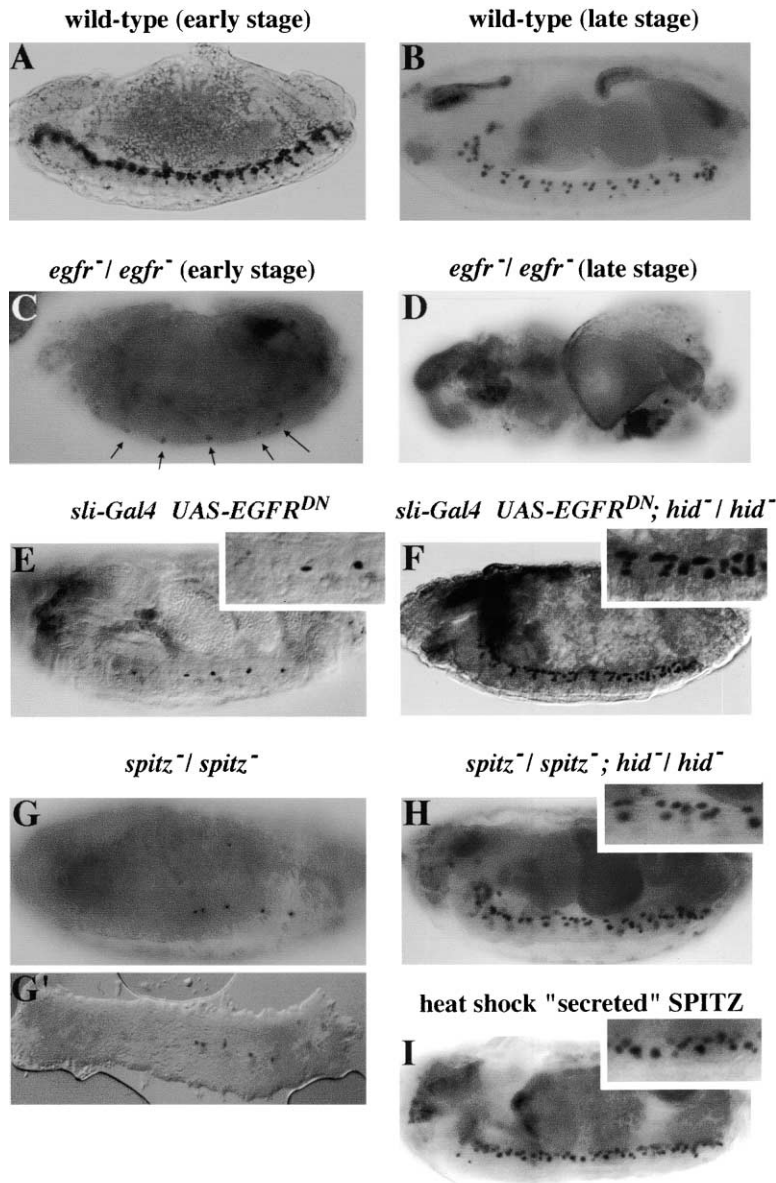


Figure 4. SPITZ/EGFR-Mediated Survival of the MG Is through Inhibition of HID

In *egfr*<sup>-</sup> mutant embryos, the MG start forming (see arrows in [C]), but the full set of MG is never generated due to severe developmental defects in these embryos (compare the wild-type embryos in [A] and [B] with the *egfr*<sup>-</sup> embryos in [C] and [D] at early stages [A] and [C] and late stages [B] and [D]). To bypass the developmental defects in *egfr*<sup>-</sup> embryos, a dominant-negative allele of the EGFR, *EGFR<sup>DN</sup>*, was expressed in the MG using the *sli-GAL4* driver in otherwise wild-type embryos. This treatment results in ablation of most of the MG (E). The MG death in response to expression of *EGFR<sup>DN</sup>* is due to a failure to suppress HID, since the MG survives in *hid*<sup>-</sup> mutant embryos expressing the *sli-GAL4/UAS-EGFR<sup>DN</sup>* transgenes (F).

Similarly, the MG in *spitz*<sup>-</sup> mutant embryos undergo apoptosis (see [G] and the nerve cord preparation of the same embryo in [G']) because they fail to suppress HID as revealed in *spitz*<sup>-</sup>; *hid*<sup>-</sup> double mutant embryos (H), suggesting that SPITZ activity is required for MG survival to suppress HID. Expression of the activated form of SPITZ, sSPI, by heat shock treatment results in rescue of additional MG (I). This result implies that MG survival depends on sufficient quantities of the EGF ligand SPITZ.

The inserts in (E), (F), (H), and (I) show enlarged views of three segments each. The mutant alleles are *egfr*<sup>-</sup> = *flb<sup>2</sup>*, *hid<sup>MR+XI</sup>*, and *spitz*<sup>-</sup>.

Figure 3G; Klämbt et al., 1991). Consistently, expression of the *UAS-hid<sup>Ala5</sup>* transgenes and consequently ablation of the MG causes a fused commissure phenotype (Figure 3H).

In summary, this analysis demonstrates that MG survival requires suppression of HID activity by MAPK. The MAPK phosphorylation sites in HID are critical for this response, providing an important mechanism for the regulation of MG number.

#### EGF Signaling Is Required for MG Survival

Activation of MAPK usually requires activation of RAS, which in turn is activated by receptor tyrosine kinase (RTK) signaling (for a review, see Downward, 1998). Scholz et al. (1997) demonstrated that MG survival depends on RAS, which is consistent with our model. Within the embryonic CNS, the *Drosophila* homolog of

the epidermal growth factor receptor (EGFR) is specifically expressed and required for MG differentiation (Zak et al., 1990; Raz and Shilo, 1992). We analyzed the requirement of EGFR signaling for MG survival.

Due to severe developmental defects in *egfr* mutants, only a few MG start forming at stage 11, and none of them survive. Thus, it is difficult to study the requirement of the EGFR for MG survival directly. To overcome this problem, we expressed a dominant-negative mutant of the EGFR (*UAS-EGFR<sup>DN</sup>*) in the MG using the *sli-GAL4* driver in otherwise wild-type embryos. In this way, EGFR activity is specifically diminished in the MG after their generation. As expected, the MG formed normally in these embryos (data not shown). However, most of the MG die during subsequent developmental stages and only a few survive to the end of embryogenesis (Figure 4E), indicating a direct requirement of the EGFR for MG survival. To determine whether the MG death in this

experimental condition is due to failure to inhibit HID, we expressed *EGFR<sup>DN</sup>* in the MG of *hid* mutants. In this genetic background, on average 6.1 MG cells survive ( $n = 112$ ; Figure 4F), demonstrating that MG survival requires functional EGFR signaling to suppress HID activity.

The *Drosophila* genome contains at least three genes encoding putative EGF-like ligands: *spitz* (*spi*), *vein*, and *gurken* (for a review, see Schweitzer and Shilo, 1997). Each has a predicted EGF-like motif. *spi* and *gurken* encode TGF $\alpha$ -like proteins and *vein* is a neuregulin-like protein (Rutledge et al., 1992; Schnepf et al., 1996; Neuman-Silberberg and Schüpbach, 1993). Gurken is utilized only during oogenesis and is not required for MG survival (Neuman-Silberberg and Schüpbach, 1993). Likewise, MG survival in *vein* mutant embryos appears to be unaffected, indicating that *vein* function is not required for MG survival (Lanoue et al., 2000; data not shown). However, in *spi* mutant embryos, the number of surviving MG is largely reduced (Klämbt et al., 1991; Figure 4G). Nerve cord preparations of *spi* mutant embryos show that only a small subset of the MG survives (Figure 4G'). Thus, the *spi* gene is required for MG survival and encodes a candidate trophic factor for MG survival.

To prove that *spi* function is required to suppress *hid* activity, we analyzed the fate of the MG in *spi*; *hid* double mutant embryos. As shown in Figure 4H, the MG survive in *spi* embryos if *hid* is removed as well. We counted 5.7 MG on average per segment ( $n = 133$ ). Since this number is very similar to the number of surviving MG in *hid* mutant embryos alone (5.8), we conclude that the survival function of *spi* is mediated through suppression of *hid*-induced apoptosis.

The SPI ligand is produced as a membrane-bound inactive precursor (mSPI). Activation of SPI occurs via proteolytic cleavage generating a secreted form of SPI (sSPI; Schweitzer et al., 1995). Ectopic expression of sSPI via heat shock treatment results in rescue of the MG (Figure 4I). This finding is consistent with the proposed function of SPI as the survival factor for the MG. One of the main suppositions of the trophic theory is that the trophic factor is provided in limited amounts, and only a proportion of cells get sufficient trophic support to survive (see Introduction; Raff et al., 1993). The result presented here strongly supports this notion. Overexpression of the survival factor sSPI helps rescue those MG that usually do not receive enough trophic support for survival.

To further address the importance of *spi* for MG function, we analyzed the expression of known target genes of the EGFR in *spi* mutant embryos. As a marker for EGFR signaling, we used induction of an enhancer trap inserted in the gene *sprouty* (*spry ET*), which mimics its expression pattern (Hacohen et al., 1998; Reich et al., 1999). The pattern of *spry ET* and activated MAPK (dpERK) in the midline are very similar (Figures 5A and 5B). In both cases, expression in the MG (arrows) and in the longitudinal glia (LG; arrowheads) are observed. Figure 5D shows the position of the MG and LG relative to the nerve cord (compare also with Figure 7A). However, in *spi* mutant embryos, *spry ET* expression in the MG is lost even though it remains intact in the LG (Figure

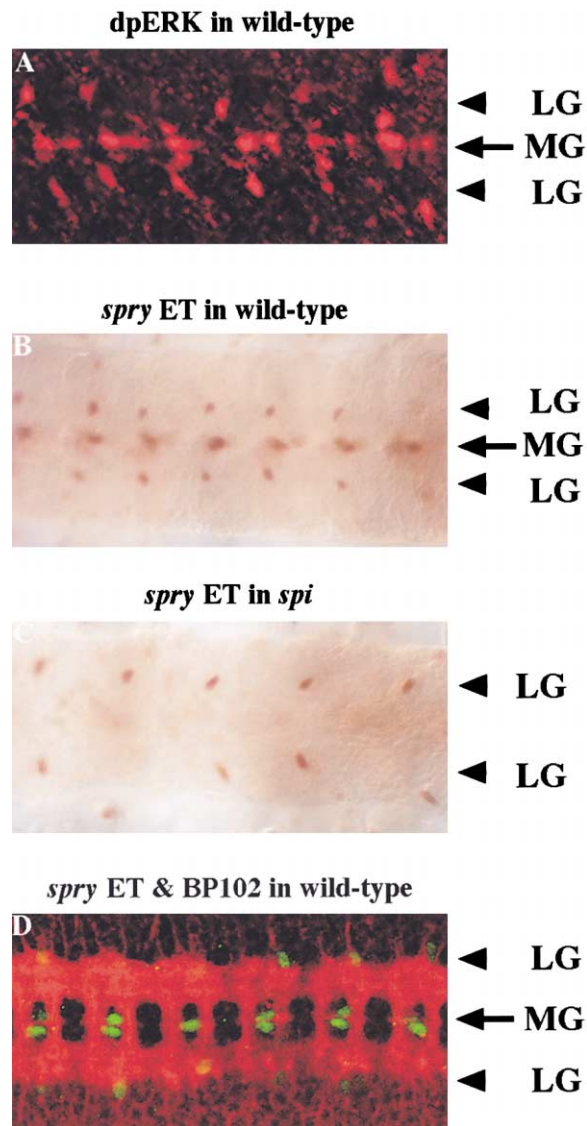


Figure 5. SPI Is the Primary Activator of EGFR Signaling in the MG (A and B) A *sprouty* enhancer trap (*spry ET*) was used as a marker of EGFR signaling in the MG. *spry ET* expression and the pattern of activated MAPK as revealed by staining with the monoclonal antibody dpERK (Gabay et al., 1997a, 1997b) in the midline are very similar in wild-type embryos. Expression is detected both in the midline glia (MG; arrow) and in the longitudinal glia (LG; arrowheads). (C) In *spi* mutant embryos, *spry ET* expression is not detected in the MG although it is still present in the LG.

(D) Double labeling of the *spry ET* and the ventral nerve cord with antibody BP102 indicates the relative position of MG undergoing EGFR signaling and neuronal axons.

5C). This analysis underscores the importance of SPI as the primary activator of the EFGR in the MG.

#### The Survival Factor sSPI Is Generated and Secreted by the Neuronal Axons

The question arises as to which cells process mSPI and provide a source of sSPI for MG survival. Since *spi* is

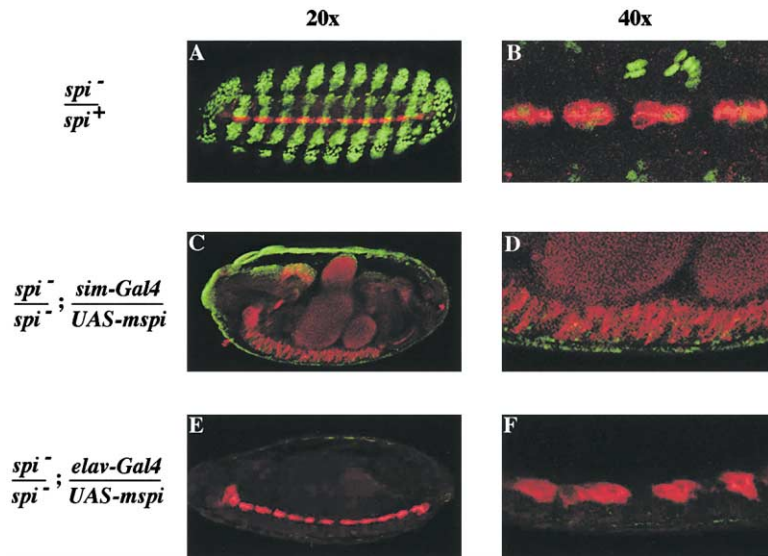


Figure 6. The Survival Factor sSPI Is Axon Derived

To determine the source of sSPI generation, we attempted to rescue the MG loss in *spi* mutant embryos (Figure 4G) by expression of the inactive membrane-bound form of SPI (*UAS-mspi*) either in the MG using the *sim-Gal4* driver (C and D) or in the neuronal axons using the *elav-Gal4* driver (E and F). We assume that mSPI will only be processed and activated to signal MG survival if it is expressed in the correct location. Embryos were stained with anti-WRAPPER antibody to visualize the MG (red; Noordermeer et al., 1999) and with anti- $\beta$ -gal antibody (green) to identify balancer-bearing embryos which are heterozygous for *spi* ([A and B]; see absence of green staining in [C]–[F]). This analysis shows that expression of *mspi* in the MG using *sim-Gal4* does not rescue MG death in *spi* mutants (C and D), suggesting that the MG is not the source of SPI processing for MG survival. In contrast, expression of *mspi* in neuronal axons using *elav-Gal4* does pro-

motivate MG survival in otherwise *spi* mutant embryos (E and F). Thus, neuronal axons can serve as the site of SPI processing for MG survival. The *spi* allele used is *spi<sup>DE92</sup>*. (A), (C), and (E) are whole-mount photographs; (B), (D), and (F) are 40 $\times$  magnifications of the same embryos.

ubiquitously expressed (Rutledge et al., 1992), it is difficult to determine histochemically where sSPI, the active ligand, is generated. Therefore, we used a genetic approach and examined whether the loss of MG in *spi* mutant embryos (see Figure 4G) can be rescued by expression of *UAS-mSPI* either in the MG (using the *sim-GAL4* driver) or in neuronal axons (using the *elav-GAL4* driver). We reasoned that the MG would be rescued in *spi* mutant embryos only if mSPI is presented in the location where it is normally processed for MG survival in wild-type embryos. Presentation of mSPI by the MG itself does not result in rescue of the MG in *spi* mutant embryos (Figures 6C and 6D), ruling out an autocrine mechanism. In contrast, expression of mSPI in neuronal axons appears to be sufficient for MG survival in *spi* embryos (Figures 6E and 6F). This argues in favor of a paracrine mechanism. In control experiments, we expressed sSPI using these two Gal4 drivers in wild-type embryos. With both GAL4 drivers an increase in the number of MG cells is detected (data not shown), indicating that they are expressed at the right time and that the MG does not fail to secrete SPI once it has been processed.

A key regulator of SPI activation is *rhomboïd*, a gene encoding a cell surface, seven-pass transmembrane protein (Bier et al., 1990) that appears to function as a serine protease directly cleaving mSPI (Urban et al., 2001). *rhomboïd* has been implicated in suppression of MG apoptosis (Lanoue and Jacobs, 1999). Ectopic expression of RHOMBOID in neurons (*elav-Gal4/UAS-Rhomboïd*) promotes an excess of MG (data not shown), suggesting that neurons have the capacity to process endogenous mSPI. Another essential protein for SPI processing is STAR (Kolodkin et al., 1994). *Star* mutants display a similar MG phenotype as *spi* (Klambt et al., 1991). STAR regulates intracellular trafficking of mSPI (Lee et al., 2001; Tsuya et al., 2002). Expression of *Star* from the neurons but not from the MG rescues the *Star*

phenotype in the MG (data not shown). Thus, this analysis clearly demonstrates that the sSPI signal for MG survival is generated and secreted by neurons.

#### Axon Contact Is Required for Activation of MAPK to Suppress HID-Induced Apoptosis

Sonnenfeld and Jacobs (1995) noted that the surviving MG in late stage embryos are in close contact to commissural axons (Figure 6A). In embryos lacking the *commissureless (comm)* gene, the commissural axons are absent (Seeger et al., 1993). In *comm* embryos the MG die prematurely, and some survivors become misplaced laterally along the longitudinal axon tracts (Figure 6B; Sonnenfeld and Jacobs, 1995). The location of the MG along the longitudinal axons in *comm* mutant embryos as well as their close contact to commissural axons in wild-type embryos prompted Sonnenfeld and Jacobs (1995) to suggest that axon contact is required for MG survival. Axon contact appears to permit the MG to respond to trophic signaling which is necessary for its survival. Consistent with this notion, using *spry ET* expression as a marker, we found that trophic signaling provided by sSPI/EGFR is present only in MG associated with longitudinal axons in *comm hid* mutants (Figure 7E). Thus, we asked whether axon contact-mediated EGFR signaling in the MG is required to activate MAPK, which in turn suppresses the cell death-inducing ability of HID.

To address this question, we analyzed the fate of the MG in *comm* mutant embryos which are at the same time mutant for *hid* (*comm hid* double mutants) or carry the dominant active *mapk* allele, *mapk<sup>Sem</sup>* (*mapk<sup>Sem</sup>*; *comm* double mutants). Strikingly, a substantial number of the MG survive even in the absence of axonal contact if *hid* function is removed or if MAPK is activated. This strongly suggests that axon contact is necessary to suppress HID via MAPK (Figures 7C and 7D). Interestingly, the analysis of *spry ET* expression in *comm hid*

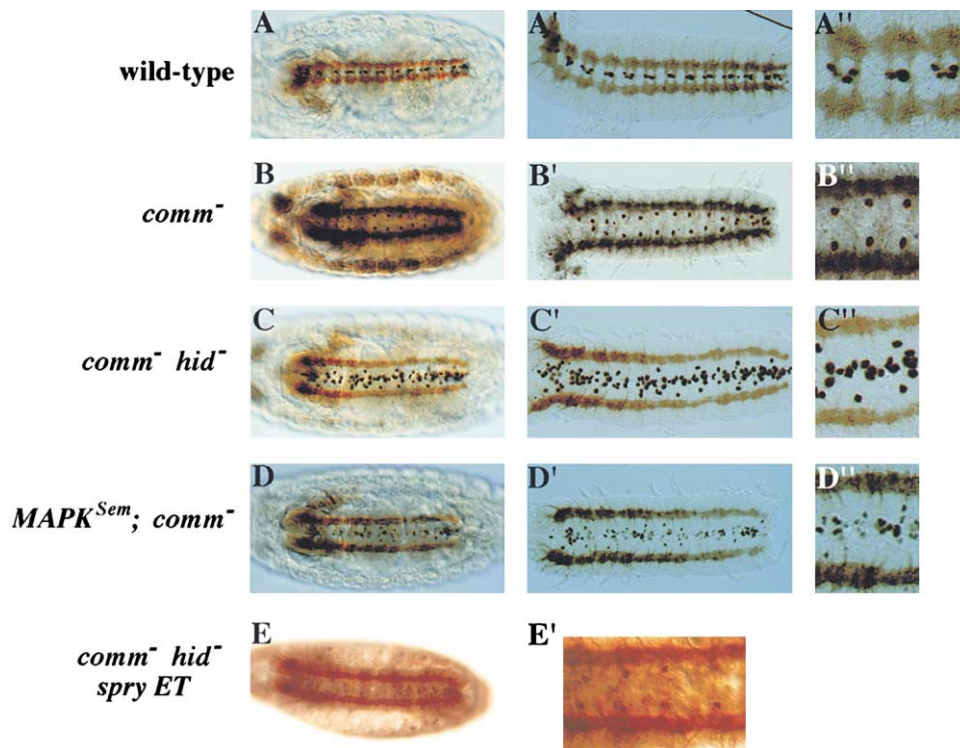


Figure 7. Axon Contact Appears to Be Required for MAPK Activation and HID Suppression

Embryos were double stained using antibody BP102 to label the CNS axon pattern (brown) and the anti- $\beta$ -gal antibody to either visualize the MG (black; [A–D]) or to visualize the *spry ET* (E). (A)–(E) are ventral views of whole-mount embryos, (A')–(D') are nerve cord preparations of the same embryos, and (A'')–(D'') show enlarged details of three segments.

(A, A', and A'') Final MG survival correlates with axon contact. The surviving MG appear to require direct contact to commissural axon tracts. See enlargement in (A'').

(B, B', and B'') In *commissureless* (*comm*<sup>-</sup>) embryos, the commissural axon tracts do not form. The MG are not present along the midline in *comm*<sup>-</sup> mutant embryos and line up along the longitudinal axon tracts, providing further evidence that MG survival requires axon contact (Sonnenfeld and Jacobs, 1995).

(C, C', and C'') In *comm*<sup>-</sup> *hid*<sup>-</sup> double mutant embryos, the MG do not require axon contact for survival.

(D, D', and D'') In *mapk<sup>Sem</sup>; comm*<sup>-</sup> double mutant embryos, the MG do not require axon contact for survival.

(E and E') The *spry ET* as a marker for EGFR signaling was analyzed in *comm hid* double mutant embryos. This analysis indicates that MG in close proximity to axons undergo EGFR signaling. MG that survive independent of axonal contact in *comm hid* embryos (C) do not undergo EGFR signaling and survive only because *hid* is mutant.

double mutants (Figure 7E) indicates that only MG in proximity to neurons undergo EGFR signaling. The additional MG that survive along the midline in *comm hid* mutants (Figure 7C) do not express *spry*, that is, do not receive an EGFR signal, and survive only because *hid* is absent in this experimental condition.

## Discussion

The elimination of cells has long been recognized as an integral part of normal animal development. Substantial progress has been made in understanding the molecular pathways that execute the cell death program (Hengartner, 2000). However, the mechanisms that regulate the activation of the cell death pathways in the context of a developing organism are largely unknown. The classical neurotrophic theory describes the survival of neurons during the time when they innervate target cells. During this period, neurons need signals from other cells, usually target cells they innervate; in the absence of such signals, they kill themselves by activating an intrinsic

suicide program. Dependence on signals from other cells for survival is not confined to neurons. There are numerous examples reviewed by Raff (1992) that list other cell types requiring signals from neighboring cells for survival.

In this paper, we use the embryonic midline glia (MG) of *Drosophila melanogaster* as a model to study trophic signaling genetically. The MG represent a group of transient cells that are associated with the establishment, morphogenesis, and ensheathment of commissural axon tracts (KlÄmbt et al., 1991; Jacobs, 2000). About ten MG per segment are generated by stage 13 of embryogenesis. The initial reduction from ten to six MG requires the function of the cell death genes *reaper* and *grim* (Zhou et al., 1997). The live-or-die decision of the remaining six MG is dependent on a trophic mechanism that involves inhibition of the cell death inducer HID by active MAPK, and is the subject of this paper. The EGFR/RAS/MAPK pathway appears to have two separate functions during MG development. In an early function, this pathway controls MG differentiation through activation



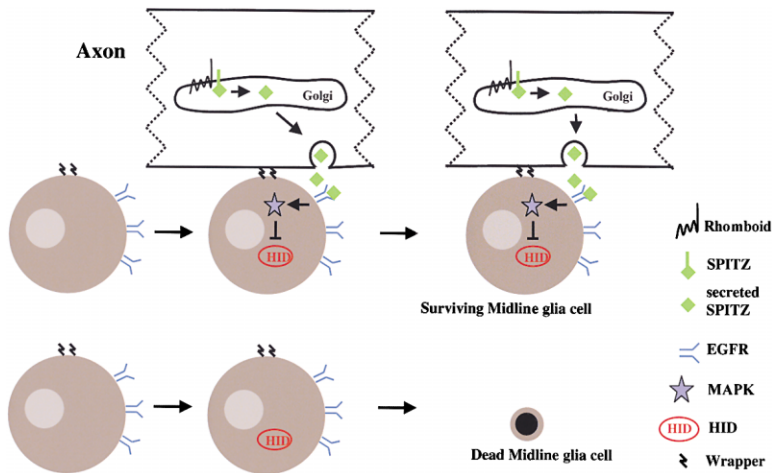


Figure 8. Model of MG Survival

The data presented in this paper are consistent with a paracrine model of MG survival (see Discussion). Two identical MG cells are shown, one of which (the upper one) comes in close contact with a neuronal axon. Cell-cell contact is established using the cell adhesion molecule WRAPPER. This brings the MG cell into close proximity to the source of the survival factor sSPI, which is secreted by the axon in this model. Generation of sSPI occurs via proteolytic cleavage mediated by RHOMBOID in the Golgi apparatus of the axon. sSPI binds to and activates the EGFR, which in turn activates the RAS/MAPK pathway. MAPK inhibits the apoptosis-inducing activity of HID by phosphorylation. Thus, this cell suppresses the intrinsic cell death program and survives. The second MG cell (the lower one) is too far away from the neuronal axon to receive sufficient quantities of the survival factor SPITZ. It fails to suppress the activity of HID and undergoes HID-induced apoptosis.

of the downstream Ets-type transcription factor *pointed* (Klämbt, 1993; Scholz et al., 1997). At later stages, when the MG ensheath commissural axons, the EGFR/RAS/MAPK pathway promotes MG survival. We show that engagement of the EGFR/RAS/MAPK pathway by the axon-derived trophic factor SPITZ mediates MG survival by blocking the apoptotic activity of HID.

#### Suppression of HID by Active MAPK Is Necessary for MG Survival

The relative levels of MAPK activity determine the extent of MG survival. Embryos carrying a dominant active MAPK allele (*mapk<sup>Sem</sup>*) contain on average six MG per segment. A similar number of surviving MG was observed by removing inhibitory components of the EGFR/RAS pathway such as *gap1*, *sprouty*, or *argos* (data not shown; Kramer et al., 1999; Stemerink and Jacobs, 1997). The gain-of-function character of *mapk<sup>Sem</sup>* is caused by a single amino acid substitution (Brunner et al., 1994) that renders the *mapk<sup>Sem</sup>* gene product resistant to inactivation (Karim and Rubin, 1999). Thus, once the MAPK<sup>Sem</sup> protein has been activated it remains activated. Initial activation of MAPK occurs early in MG development when it is required for MG differentiation (Scholz et al., 1997; Jacobs, 2000). We detect active MAPK using the dpERK antibody as early as stage 14 (data not shown; Gabay et al., 1997b).

Since the MAPK<sup>Sem</sup> product is resistant to inactivation, the analysis of *mapk<sup>Sem</sup>* embryos suggests that there is a group of about six MG cells whose survival depends upon MAPK activity. Within this group, the final number of surviving MG cells in wild-type embryos is determined by a mechanism that involves inhibition of the cell death inducer HID by active MAPK. This conclusion is based on a number of observations. First, in *mapk* null mutants, all of the MG undergo apoptosis. However, in *mapk; hid* double mutant embryos, the MG fail to die. This result strongly suggests that MG survival requires inhibition of HID by MAPK. Second, the number of surviving MG in *hid* mutant embryos is very similar to that in *mapk<sup>Sem</sup>*

embryos (5.8 in *hid* mutants versus 6.0 in *mapk<sup>Sem</sup>* embryos). Third, the *mapk<sup>Sem</sup>; hid* double mutant analysis implied that activation of MAPK and expression of *hid* occurs in largely the same MG cells. Fourth, expression of *hid<sup>Ala5</sup>*, a MAPK-unresponsive mutant of *hid*, results in MG apoptosis, suggesting that inhibitory phosphorylation of HID by MAPK is required for MG survival. Thus, the final number of surviving MG is determined by competitive interaction between MAPK and HID.

#### MG Survival Is Dependent on a Trophic Mechanism

There are three distinct glial-neuronal interactions at the midline. First, the MG provide guidance cues for commissural growth cones; second, separation of the anterior and posterior commissural axon tracts requires the function of the MG; finally, the MG ensheath separated commissural axons (Klämbt et al., 1991; Jacobs, 2000). Genetic evidence has indicated that the ensheathing MG require axon contact for survival (Sonnenfeld and Jacobs, 1995; Noordermeer et al., 1999). This is based on the observation that in *commisssureless (comm)* mutant embryos in which the commissural axon tracts are absent, the MG fail to make sufficient axon contact and die (Sonnenfeld and Jacobs, 1995). Surviving MG in *comm* embryos have migrated laterally, presumably to receive trophic support from longitudinal axons (Sonnenfeld and Jacobs, 1995; Figures 6B and 6E). Furthermore, ensheathment of axons by the MG requires the function of the gene *wrapper*, an Ig domain-type protein that is expressed on the surface of the MG (Noordermeer et al., 1999). WRAPPER promotes cell-cell contact between axons and the MG. In the absence of *wrapper*, ensheathment does not occur, thereby reducing cell contact between the MG and the commissural axons. This lack of axon contact correlates with increased MG death (Noordermeer et al., 1999).

MG apoptosis in *comm* mutant embryos can be prevented either if these embryos are mutant for *hid* (i.e., *comm hid* double mutants), or if MAPK is activated in

*comm* embryos (*mapk<sup>sem</sup>*; *comm* double mutants). Thus, by either removing *hid* or by activating MAPK, axon contact is no longer required for MG survival in *comm* mutant embryos. This analysis and the genetically defined competitive interaction between *mapk* and *hid* imply that in wild-type embryos, glial-axonal interaction is required to maintain active MAPK, which in turn suppresses the cell death inducer HID. We conclude that trophic signaling promotes survival of the MG.

Axon contact appears to permit the MG to receive sufficient quantities of the trophic factor Spitz (SPI), a TGF $\alpha$ -like factor with an EGF motif that binds to and activates the EGFR. In *spi* mutants or in embryos expressing a dominant-negative EGFR the MG undergo apoptosis, and these deaths require *hid*, based on double mutant analysis. *spi* is expressed as an inactive membrane-bound precursor (mSPI) which requires proteolytic processing in order to generate the active, secreted form of SPI, sSPI. The loss of MG in *spi* mutants can be rescued when mSPI is expressed selectively in neurons (Figure 6), suggesting that generation of sSPI occurs at the neuronal axons, thus favoring a paracrine mechanism of SPI activation. Furthermore, overexpression of sSPI promotes survival of additional MG, suggesting that sSPI is available in limited quantities for MG survival. Taken together, these data strongly suggest that SPI functions for MG survival as a trophic factor derived from neighboring axons. It is interesting to note that another group of glia cells, the longitudinal glia, requires a different EGFR ligand for its survival. In this case, the *Drosophila* neuregulin homolog *vein* is produced by neighboring pioneer neurons and maintains the survival of longitudinal glia (Hidalgo et al., 2001).

The results presented here extend previous models about MG survival (Sonnenfeld and Jacobs, 1995; Noordermeer et al., 1999). After commissure separation is completed, some of the MG start ensheathing them. This process requires the function of the gene *wrapper*, which appears to permit MG cells to have intimate contact with commissural axons. MG that have this intimacy survive because they receive sufficient quantities of the trophic survival factor sSPI. Proteolytic processing of mSPI requires the function of *Star* and *rhomboid*. Recent data have demonstrated that *Star* regulates intracellular trafficking of mSPI from the ER to the Golgi apparatus, where *Rhomboid* promotes cleavage of mSPI (Lee et al., 2001; Tsruya et al., 2002; Urban et al., 2001). The activated ligand sSPI is then directly secreted (Figure 8). sSPI activates the EGFR pathway in the MG, resulting in MAPK activation. Active MAPK in turn suppresses HID by phosphorylation, and the MG survive (Figure 8).

We noted that active MAPK is capable of rescuing a total of six MG based on analysis of *mapk<sup>sem</sup>* embryos (Figure 2C). Presumably, this MAPK activation in *mapk<sup>sem</sup>* embryos is inherited from the differentiation period of the MG. However, only three MG survive by stage 17 in wild-type embryos. We propose that of the group of six MG that require MAPK for survival, only the three surviving cells make adequate axon contacts necessary to receive sufficient quantities of the survival factor sSPI. According to this model, the remaining three MG die because they lose the competition for axon contact and do not receive levels of sSPI that are high enough to

inactivate HID via phosphorylation by MAPK. If additional sSPI is provided in the midline, additional MG can be rescued. The limited availability of axon-derived sSPI would serve to match the number of MG to the length of commissural axons requiring ensheathment. Thus, the regulation of MG number and survival represents a genetically defined example of the classical trophic theory of cell survival.

## Conclusion

The regulation of MG apoptosis in *Drosophila* bears striking overall similarity to the regulation of glial cell death in the rat optic nerve. Raff et al. (1993) described an early dependence of the oligodendroglia in the rat optic nerve on growth factors for differentiation followed by a dependence on axon contact for survival. However, it is not clear how the oligodendroglia in the rat optic nerve survive upon axon contact. Since mammalian homologs for many of the components in the apoptotic pathway both upstream and downstream of *Drosophila* HID are known, it will be interesting to analyze whether similar molecules regulate apoptosis and cell number in the mammalian nervous system. Therefore, molecular genetic studies in *Drosophila* promise considerable insights for advancing our understanding of the basic control mechanisms involved in the regulation of apoptosis in the context of a developing organism in vivo.

## Experimental Procedures

The following mutant and transgenic fly strains were used: P(*slit1.0-lacZ*) (Wharton and Crews, 1993), *rl/mapk<sup>10a</sup>* (Peverali et al., 1996), *rl/mapk<sup>AL528</sup>* (Rebay et al., 2000), *rl<sup>sem</sup>/MAPK* (Brunner et al., 1994), *egfr = flb<sup>12</sup>, spi<sup>1</sup>*, and *sp<sup>10E92</sup>* (Tearle and Nüsslein-Volhard, 1987), *Df(3L)H99*, *hid<sup>WR+X1</sup>*, and *hid<sup>A329</sup>* (Abbott and Lengyel, 1991), *comm<sup>3e39</sup>* (Kidd et al., 1999), *comm<sup>3e39</sup> hid<sup>A329</sup>* (this study), *slit1.0-GAL4* and *sim-GAL4* (Scholz et al., 1997), *UAS-hid<sup>+</sup>* (line 14; Zhou et al., 1997), *UAS-hid<sup>Alas5</sup>* (lines 44 and 49; this study), *UAS-EGFR<sup>DN</sup>* (O'Keefe et al., 1997), and *UAS-sspi4a* and *UAS-mspi7b* (Schweitzer et al., 1995). *mapk*-deficient females were obtained following a heat shock procedure described by Biggs et al. (1994) using the *mapk* alleles *rl<sup>10a</sup>* and *rl<sup>AL528</sup>*. The monoclonal anti-Wrapper antibody is a kind gift of Jasperien Noordermeer.

The P(*slit1.0-lacZ*) reporter transgene was crossed in various mutant backgrounds, and the fate of the MG was analyzed by  $\beta$ -gal immunohistochemistry following standard protocols (Patel, 1994). The identification of homozygous mutant embryos was facilitated by the use of blue balancer chromosomes. The number of MG was averaged for segments T2 to A5. "n" denotes the total number of segments.

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