Regulation of *Drosophila* glial cell proliferation by Merlin-Hippo signaling

B. V. V. G. Reddy and Kenneth D. Irvine*

**SUMMARY**

Glia perform diverse and essential roles in the nervous system, but the mechanisms that regulate glial cell numbers are not well understood. Here, we identify and characterize a requirement for the Hippo pathway and its transcriptional co-activator Yorkie in controlling *Drosophila* glial proliferation. We find that Yorkie is both necessary for normal glial cell numbers and, when activated, sufficient to drive glial over-proliferation. Yorkie activity in glial cells is controlled by a Merlin-Hippo signaling pathway, whereas the upstream Hippo pathway regulators Fat, Expanded, Crumbs and Lethal giant larvae have no detectable role. We extend functional characterization of Merlin-Hippo signaling by showing that Merlin and Hippo can be physically linked by the Salvador tumor suppressor. Yorkie promotes expression of the microRNA gene *bantam* in glia, and *bantam* promotes expression of Myc, which is required for Yorkie and *bantam*-induced glial proliferation. Our results provide new insights into the control of glial growth, and establish glia as a model for Merlin-specific Hippo signaling. Moreover, as several of the genes we studied have been linked to human gliomas, our results suggest that this linkage could reflect their organization into a conserved pathway for the control of glial cell proliferation.

**KEY WORDS:** *Drosophila*, Hippo, Merlin, Glia

**INTRODUCTION**

Neurons and glia are two distinct cell types that together form the nervous system. Glial cells perform diverse essential functions to support and protect neurons, and to guide and maintain their connections (Freeman and Doherty, 2006; Barres, 2008). Despite their fundamental importance, the mechanisms that control glial cell numbers during development are not well understood. Moreover, cancers associated with over-proliferation of glial cells (gliomas) include the most common and deadly type of brain tumor in adults (glioblastoma) and the most common solid tumors in children. A number of different oncogenes and tumor suppressors have been implicated in gliomas, including activation of receptor tyrosine kinase pathways, activation of phosphoinositide 3 (PI3) kinase signaling, activation of transforming growth factor-β (TGF-β) signaling, elevation of Myc levels, expression of microRNAs and loss of the tumor suppressor merlin (Ruttledge et al., 1994; Herm et al., 1999; Maher et al., 2001; Lassman, 2004; Furnari et al., 2007; Lau et al., 2008; Zheng et al., 2008; Abounader, 2009; Godlewski et al., 2009; Silber et al., 2009). A better understanding of the relationships among distinct genetic lesions associated with gliomas could facilitate rational, targeted approaches to diagnosis and treatment.

Human merlin is the product of the neurofibromatosis type 2 (neurofibromin 2, *NF2*) locus, a familial cancer syndrome in which afflicted individuals develop tumors of the peripheral nervous system, especially benign schwannomas and meningiomas, and malignant mesotheliomas (Astagiri et al., 2009). Merlin is a member of the ezrin/radixin/moesin (ERM) protein family, which link the actin cytoskeleton to membrane proteins (Fievet et al., 2007). Gene-targeted mutations in murine merlin (neurofibromatosis 2 – Mouse Genome Informatics), together with experiments in cultured cells, have implicated it in a broad spectrum of tumor biology, as knockout mice are prone to a range of metastatic tumors, and merlin is required for contact inhibition in cultured cells (McClatchey and Giovannini, 2005; Stamenkovic and Yu, 2010). The action of merlin as a tumor suppressor has been linked to several pathways and processes. One important link, first identified in *Drosophila*, is to the Hippo signaling pathway (Hamaratoglu et al., 2006).

Hippo signaling is a recently discovered pathway that controls organ growth from *Drosophila* to humans (Fig. 1A) (Reddy and Irvine, 2008; Pan, 2010; Zhao et al., 2010; Halder and Johnson, 2011). Hippo signaling is transduced through transcriptional co-activator proteins, known as Yap and Taz in mammals, and Yorkie (Yki) in *Drosophila*, which regulate the expression of genes important for growth, cell cycle progression and inhibition of apoptosis (Oh and Irvine, 2010). Yki/Yap act in concert with DNA-binding partner proteins, which in *Drosophila* include Scalloped (Sd), Homothorax (Hth) and Mad (Oh and Irvine, 2010; Oh and Irvine, 2011). The transcriptional activity of Yki/Yap is negatively regulated by the kinase Warts (Lats in mammals), which affects Yki/Yap levels and localization. In *Drosophila*, the activity and localization of Wts is regulated through multiple upstream branches (Reddy and Irvine, 2008; Staley and Irvine, 2011). The two best-studied branches are Fat-Hippo signaling, which involves a cadherin family protein called Fat, and Expanded-Hippo signaling, which involves a FERM protein related to Merlin, called Expanded (Ex). In *Drosophila* organs studied to date, Merlin has only modest effects on Hippo signaling, apparently because of partial redundancy with Ex (McCartney et al., 2000; Hamaratoglu et al., 2006).

Studies in cultured mammalian cells, and more recently in mice, support the existence of a merlin-hippo pathway in mammals, and its importance to tumors associated with loss of merlin (Zhao et al.,

Howard Hughes Medical Institute, Waksman Institute and Department of Molecular Biology and Biochemistry, Rutgers The State University of New Jersey, Piscataway, NJ 08854, USA.

*Author for correspondence (irvine@waksman.rutgers.edu)
2007; Striedinger et al., 2008; Zhang et al., 2010; Zhao et al., 2010). Despite this, the extent to which mammalian merlin functions as a tumor suppressor through hippo signaling has remained unclear, as merlin has been linked to diverse downstream effectors (Rong et al., 2004; Maitra et al., 2006; Morrison et al., 2007; Striedinger et al., 2008; Houshmandi et al., 2009; Lopez-Lago et al., 2009; Li et al., 2010; Stamenkovic and Yu, 2010; Zhang et al., 2010) and recent studies disagree about whether the tumor suppressor function of merlin in liver is mediated through Yap or through epidermal growth factor receptor (EGFR) signaling (Benhamouche et al., 2010; Zhang et al., 2010).

The *Drosophila* central nervous system (CNS) comprises a ventral nerve cord and two brain hemispheres. Recent studies have begun to investigate *Drosophila* as a model for glial cell proliferation and have shown that activation of some of the signaling pathways implicated in human glioma, including EGFR, TGF-β and PI3 kinase, can also increase glial cell numbers in *Drosophila* (Rangarajan et al., 2001; Klambt, 2009; Read et al., 2009; Witte et al., 2009). The arrangement and morphology of glial cells in the CNS complicates analysis of glial proliferation. However, the developing *Drosophila* eye has also been used as a model for glial cell development (Silies et al., 2010). Retinal glial cells originate from the optic stalk, which connects the eye imaginal disc to the brain, and then migrate into the eye disc as photoreceptor cells differentiate (Fig. 1B). The arrangement and accessibility of retinal glial cells and their migration into the eye disc offers an accessible system to study glial cell proliferation and migration.

Here, we analyze the contribution of the Hippo signaling pathway to the control of glial cell proliferation in *Drosophila*, using both the brain hemispheres and the eye disc as models. We show that Merlin has a conserved role in controlling glial proliferation from humans to *Drosophila*, and that its effects in *Drosophila* can be accounted for by modulation of Hippo signaling. Unlike previously examined tissues in *Drosophila*, in glial cells Hippo signaling is controlled exclusively through a Merlin-Hippo pathway and other upstream regulators have no detectable role. We also establish that Yki activation is not only sufficient to promote glial overgrowth, but also essential for normal glial growth during nervous system development. Characterization of the downstream regulatory pathways through which Yki acts reveals that Yki functions with at least two different DNA-binding partners, and regulates glial growth, at least in part, through the microRNA gene *bantam* (*ban*), and that ban in turn acts in part through the oncogene *Myc*. Our results define a regulatory pathway that controls glial cell numbers in *Drosophila*, which includes genes implicated in human gliomas, suggesting that the regulatory links we identify and their importance to glial growth could be conserved.

**MATERIALS AND METHODS**

**Drosophila genetics**

Expression of UAS lines in glia was achieved by crossing to *repo-Gal4 UAS-mCD8:GFP* flies (gift of J. Thomas, Salk Institute, San Diego, USA). Expression of UAS lines in clones was achieved by crossing to *repo-Gal4 UAS-mCD8:GFP* (AyGal4). Expression of UAS lines in glia was achieved by crossing to *Drosophila*, and that its effects in *Drosophila* were investigated with gene anti-Repo (1-400), rat anti-Elav (1-400, Developmental Studies Hybridoma Bank (DSHB)), rabbit anti-Yki (1-400), guinea pig anti-dMyc (1-200, gift of G. Morata, Universidad Autonoma de Madrid, Spain), guinea pig anti-Merlin (gift of R. Fehon, University of Chicago, USA), goat anti-gal-4 (1-1000, Biogenesis), rabbit anti-Hh (1-400, gift of A. Salzberg, Israel Institute of Technology, Haifa, Israel) and mouse anti-Deep1 (1-200, B. Hay, California Institute of Technology, Pasadena, USA).

**Histology and imaging**

Tissue was fixed and stained as described previously, using mouse anti-Repo (1-400), rat anti-Elav (1-400), Developmental Studies Hybridoma Bank (DSHB), rabbit anti-Yki (1-400), guinea pig anti-dMyc (1-200, gift of G. Morata, Universidad Autonoma de Madrid, Spain), guinea pig anti-Merlin (gift of R. Fehon, University of Chicago, USA), goat anti-gal-4 (1-1000, Biogenesis), rabbit anti-Hh (1-400, gift of A. Salzberg, Israel Institute of Technology, Haifa, Israel) and mouse anti-Deep1 (1-200, B. Hay, California Institute of Technology, Pasadena, USA).

**RESULTS**

**Expression of Yki in glial cells**

We recently described a role for Yki in controlling the proliferation and differentiation of neuroepithelial cells in the *Drosophila* optic lobe (Reddy et al., 2010). In the course of this study, we noticed that Yki is also expressed in glial cells. This glial expression is evident as a distinctive meshwork of Yki staining throughout the central brain (Fig. 1C). The assignment of this Yki staining to glial cells was confirmed by two observations. First, Yki expression overlaps the glial-specific expression of the reversed polarity (repo) gene (Xiong et al., 1994), as revealed by a repo-Gal4 line driving the expression of a GFP transgene (*UAS-mCD8:GFP*) (Fig. 1C).
Second, when RNAi-mediated downregulation of Yki was targeted to glial cells, using repo-Gal4 to drive the expression of a yki hairpin transgene (RNAi-yki), the meshwork staining of Yki in the central brain was lost, whereas Yki expression in neuroepithelial cells of the optic lobe was unaffected (Fig. 1D). These same experiments identified Yki expression within glial cells of the ventral nerve cord (VNC), eye disc and optic stalk (Fig. 1E-H).

**Yki regulates glial cell proliferation**

To investigate the role of Yki in glia, we took advantage of the glial-specific depletion of Yki in repo-Gal4 RNAi-yki flies. These animals can reach pupal stages, but never survive to adulthood, indicating that the activity of Yki in glial cells is essential for viability [yki mutants die as first instar larvae (Huang et al., 2005)]. Examination of neural tissues revealed that the brain and VNC are reduced in size and have fewer glial cells, as identified by Repo antibody staining (Fig. 2A,B,D,E; supplementary material Fig. S1A,B). We first quantified the reduction in glial cells within the eye imaginal disc. In wild type, the number of glial cells detected in the eye disc is correlated with photoreceptor cell differentiation. In late third instar larvae with ten rows of photoreceptor cells specified (as defined by staining with the neuronal antigen Elav), wild-type eye discs had on average 105 glial cells, whereas repo-Gal4 RNAi-yki eye discs averaged 66 glial cells (Fig. 2D,E,H). Quantification also confirmed a reduction in glial cell numbers within the brain and even a decrease in total brain volume (Fig. 2I). These observations establish that Yki is required for the normal proliferation and/or survival of glial cells.

To investigate whether Yki is not only necessary for normal glial growth, but also sufficient to drive over-proliferation of glial cells, we used repo-Gal4 to express wild-type and activated forms of Yki in glial cells. Expression of a wild-type transgene (UAS-yki:V5) (Oh and Irvine, 2009) did not significantly affect glial cell numbers (Fig. 2F). However, expression of a Yki isoform activated by mutation of a key Wts phosphorylation site (UAS-ykiS168A:V5) (Oh
and Irvine, 2009) induced a substantial increase in glial cells, and a corresponding increase in the size of the VNC and brain hemispheres (Fig. 2C,G,I; supplementary material Fig. S1C). The increase in glial cell numbers was hard to discern by Repo staining in cross sections through the VNC and central brain, presumably owing to the dispersed nature of glial cell nuclei. However, it was clearly evident in projections through the optic stalk, which becomes much thicker and contains many more glial cells (Fig. 2G), and also by the increased number of glial cell nuclei in optical sections through the outer cortex of the brain hemispheres (Fig. 2C). Using image-analysis software, we were able to confirm an increase in total glial cell numbers throughout the brain, as well as an increase in total brain volume (Fig. 2I). Labeling for cells in S phase with EdU confirmed that expression of activated-Yki is associated with increased retinal glial cell proliferation (Fig. 2J,K). Under similar conditions, EdU labeling of central brain glia was observed only rarely, both in wild type and in animals expressing activated-Yki (supplementary material Fig. S1D,E). We surmise that at late third instar most non-retinal glial cells are insensitive to activated-Yki, and that the measurable increase in glial cells within the central brain reflects the cumulative effect of Yki activation throughout development.

Fig. 2 Influence of Yki on glial growth. (A-C') Drosophila brain lobes, at the same magnification, stained for glial nuclei with anti-Repo (red) from larvae expressing repo-Gal4 UAS-dcr2 UAS-mCD8:GFP (green) alone (A, control) or with UAS-RNAi-yki (B) or UAS Yki:V5S168A (C). (D-G') Third instar eye discs, stained for Repo (red) and Elav (blue), from larvae expressing repo-Gal4 UAS-mCD8:GFP (green) alone (D, control) or with UAS-RNAi-yki (E), UAS-Yki:VS (F) or UAS-Yki:V5S168A (G). (H) Histogram showing average number of glial nuclei per eye disc in larvae expressing the indicated UAS-RNAi or over-expression transgenes, under repo-Gal4 control. (I) Histograms showing average number of glial nuclei per brain hemisphere (blue, left) or average brain hemisphere volume (green, right, normalized to the wild-type average) in larvae expressing UAS-RNAi-yki or UAS-Yki:VS168A, as indicated, under repo-Gal4 control. (J-K') Third instar eye discs, stained for Repo (red) and EdU (cyan), from larvae expressing repo-Gal4 alone (J, control) or with UAS-Yki:VS168A (K). A’-G’,J,K’ show a single channel of the stain to the left. Error bars represent s.d.
A Merlin-Hippo pathway regulates glial cell numbers through Yki

Yki is the transcriptional effector of the Hippo signaling pathway (Oh and Irvine, 2010). One of the upstream regulators of the Hippo pathway is Merlin, which was first identified as a tumor suppressor gene in humans through its influence on the proliferation of peripheral glial cells (Gusella et al., 1996). RNAi-mediated depletion of Merlin in glial cells substantially increased glial cell numbers in both the optic stalk and the brain cortex (Fig. 2H, Fig. 3A; supplementary material Fig. S2A). Expression of a dominant-negative form of Merlin also increased glial cell numbers (Fig. 2H, Fig. 3C,D; supplementary material Fig. S2C,D). Kibra was recently identified as a protein that interacts with Merlin and modulates Hippo pathway activity (Genevet et al., 2010; Ling et al., 2010; Yu et al., 2010); depletion of Kibra also increased glial cell numbers (Fig. 2H, Fig. 3E). These observations, together with the consequences of expression of activated-Yki, indicate that depletion of Merlin and inactivation of the Hippo pathway result in similar glial overgrowth phenotypes.

Two additional experiments were performed to test directly the hypothesis that Merlin controls glial growth through Yki. First, we examined the subcellular localization of Yki, which is normally predominantly cytoplasmic, but which accumulates in the nucleus when upstream tumor suppressors in the Hippo pathway are mutant (Dong et al., 2007; Oh and Irvine, 2008). Depletion of Merlin, or mutation of wts, within clones of glial cells elevated nuclear localization of Yki in comparison with neighboring wild-type cells.
Thus, Merlin and Wts regulate Yki localization in glia. The functional significance of this effect on Yki localization was established by epistasis tests, which revealed that depletion of Yki suppressed the increase in glial cell numbers associated with depletion of Merlin (Fig. 2H, Fig. 3B; supplementary material Fig. S2B). Thus, the Merlin overgrowth phenotype in Drosophila glia is yki-dependent.

Genetic studies of Hippo signaling in Drosophila have identified Fat-dependent and Ex-dependent pathways as major regulators of Hippo signaling in other tissues (Reddy and Irvine, 2008; Staley and Irvine, 2011). However, in earlier studies, we noted that expression of Fat and Ex in the brain appeared to be restricted to neuroepithelial cells, whereas Merlin is expressed throughout the brain (Reddy et al., 2010). Confirmation that this ubiquitous expression of Merlin includes glial cells was provided by staining eye discs for both Merlin expression and glial cell markers (Fig. 3G). To confirm that Fat and Ex do not influence glial growth, their expression was downregulated in glial cells using RNAi lines that generate strong phenotypes in imaginal discs. RNAi of fat or ex in glial cells did not increase glial cell numbers (Fig. 2H, Fig. 3F; supplementary material Fig. S2G). As an additional test, we examined animals mutant for null alleles of fat or ex. These survive beyond late third instar, but have fewer photoreceptor cells (Feng and Irvine, 2007; Pellock et al., 2007) and we observed concordant decreases in numbers of glial cells in the eye disc and a lack of glial overgrowth (supplementary material Fig. S2H,I). We also examined two more recently identified regulators of Hpo signaling, crumbs (crb) and lethal giant larvae [lgl; l(2)gl – FlyBase] (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010; Sun and Irvine, 2011). Depletion of their expression had no detectable effect on glial cell numbers (supplementary material Fig. S2J,K). Although we cannot exclude the possibility that null alleles of crb or lgl might affect glial cells, these RNAi lines phenocopy strong mutant alleles in imaginal discs (Robinson et al., 2010; Sun and Irvine, 2011), and crb is known to act through ex, which, as noted above, has no role in glia. Thus, our observations, together with the strong effect of Merlin depletion on glial cell numbers, imply that in Drosophila glial cells Hippo signaling is regulated exclusively by a Merlin-dependent pathway.

Merlin and Ex are both FERM domain proteins, and both are thought to inhibit Yki activity principally by promoting Hippo activity. Consistent with a recent report (Yu et al., 2010), we observed that Ex, but not Merlin, could be directly co-precipitated with Hpo when expressed together in cultured Drosophila S2 cells (Fig. 3H). Although Merlin does not bind Hpo directly, both Merlin and Hpo have been reported to be able to bind to the scaffolding protein Salvador (Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Formstecher et al., 2005; Yu et al., 2010). To investigate whether Salvador might thus be able to serve as a bridge linking Hpo to Merlin, we assayed for Salvador-dependent co-precipitation of Merlin with Hpo in S2 cells. Indeed, when all three proteins were co-expressed, Merlin and Hpo were efficiently co-precipitated, and this depended upon the presence of the Hpo-binding region of Salvador (the SARAH domain) (Fig. 3H). Thus, both Ex and Mer can form complexes with Hpo, but Mer-Hpo complexes are Sav-dependent. Although the role of Salvador has previously been suggested as being to promote phosphorylation of Wts by Hpo (Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003), our observations suggest that Sav might also have a distinct role in facilitating Mer-Hippo signaling.

**Both Sd and Mad are required for Yki-promoted glial growth**

Yki functions as a transcriptional co-activator protein and regulates the expression of downstream target genes in conjunction with DNA-binding partners. Three distinct Yki partners have been identified in Drosophila: Sd, Hth and Mad (for a review, see Staley and Irvine, 2011). Within imaginal discs, Sd is normally only required for growth of the wing, but it is also required more broadly for overgrowths associated with Yki over-expression (Wu et al., 2008; Zhang et al., 2008). A requirement for Hth in Yki-dependent growth has been identified in anterior eye disc cells, which do not require Sd (Peng et al., 2009). Mad is required broadly for growth in many Drosophila tissues (Affolter and Basler, 2007). This requirement for Mad reflects its role as the transcriptional effector of Dpp signaling and had been suggested to stem from its ability to repress expression of the transcriptional repressor protein Brinker (Affolter and Basler, 2007). However, recent studies indicated that Mad can also promote growth in imaginal discs in conjunction with Yki, at least in part by promoting transcription of ban (Oh and Irvine, 2011).

The requirements for each of these DNA-binding Yki partners in glial cell proliferation was assessed by RNAi-mediated depletion. These studies identified requirements for both Sd and Mad, but not Hth, in glial cell proliferation (Fig. 4A-C, Fig. 2H). The lack of effect of Hth was not due to ineffective RNAi, because antibody staining confirmed that hth RNAi could deplete Hth protein (supplementary material Fig. S3A). Similar to yki RNAi,
depletion of Sd or Mad in glial cells reduced glial cell numbers (Fig. 4A,B, Fig. 2H). Moreover, depletion of Sd or Mad suppressed the ability of activated-Yki to promote glial growth, whereas depletion of Hth did not (Fig. 2H, Fig. 4D-F).

The observation that both Sd and Mad are required for Yki-promoted glial growth indicates that, in glia, both of these DNA-binding partners are required, possibly to regulate distinct sets of downstream genes. One gene identified as a direct target of Yki-Sd regulation in imaginal discs, and not Yki-Mad regulation, is thread (th), which encodes Diap1 (Wu et al., 2008; Zhang et al., 2008; Oh and Irvine, 2011). Expression of a th-lacZ reporter was upregulated by expression of Yki S168A:V5 in glial cells (Fig. 5A), consistent with the inference that Sd is a Yki partner in glia, with th as one of its targets. Moreover, depletion of Sd, but not depletion of mad, reduced Diap1 expression in glial cells (Fig. 5B,C).

Another key downstream target of Yki in imaginal discs is the microRNA (miRNA) gene bantam (ban) (Brennecke et al., 2003; Nolo et al., 2006; Thompson and Cohen, 2006). In imaginal discs, Yki can promote ban expression in conjunction with either Sd, Hth or Mad, acting through distinct enhancers (Zhang et al., 2008; Peng et al., 2009; Oh and Irvine, 2011). ban expression can be detected using a GFP-ban sensor, which inversely reports ban expression by virtue of ban target sites in the 3’ UTR of a GFP-expressing transgene (Brennecke et al., 2003). The GFP-ban sensor is expressed at high levels in retinal axons within the optic stalk, but is barely detectable within glial cells, which implies that ban is expressed in glial cells (Fig. 5D). A further reduction of the ban sensor in glial cells could be induced by expression of activated-Yki (Fig. 5E), whereas yki RNAi upregulated ban sensor expression (Fig. 5F). Thus, ban is regulated downstream of Yki in glial cells. Investigation of requirements for Sd and Mad in glial cells revealed that mad RNAi elevated ban sensor expression (Fig. 5G), and was epistatic to Yki activation for ban regulation (Fig. 5H). Conversely, sd RNAi had no detectable effect on ban sensor expression in glia (Fig. 5I). Thus, ban is apparently regulated in glial cells through a Yki-Mad transcription factor complex, and not a Yki-Sd complex.
Regulation of glial growth by Yki through ban and Myc

To assess the influence of ban on glial growth, we took the advantage of transgenes that drive expression of ban under UAS-Gal4 control. Over-expression of ban in glial cells, under repo-Gal4 control, induced substantial over-proliferation of both retinal and central brain glia (Fig. 6B,K), even greater than that induced by activated-Yki. Conversely, ban mutant animals, which survive to the third larval instar, have greatly reduced numbers of glial cells (Fig. 6A,L). Thus, ban is a crucial growth regulator in glial cells. Ban-induced glial overgrowth is not blocked by depletion of yki (Fig. 6C). Moreover, expression of activated-Yki could not reverse the reduction in glial cell numbers associated with mutation of ban, nor it could rescue the reduced growth of ban mutants, even using a variety of Gal4 drivers (repo-Gal4, Fig. 6M; tub-Gal4 or actin-Gal4, not shown). These observations suggest that ban is the key downstream target of Yki for the promotion of glial cell proliferation.
Increased expression of the Myc oncogene has been correlated with gliomas in humans (Herms et al., 1999). Myc has also recently been identified as a direct target of Yki in imaginal discs (Neto-Silva et al., 2010), and we observed a modest upregulation of Myc when activated-Yki was expressed (Fig. 6I). ban has also been reported to upregulate Myc protein levels in wing discs, acting through downregulation of the ubiquitin ligase Mei-P26 (Herranz et al., 2010). Myc antibody staining revealed that ban could also upregulate Myc levels in glial cells (Fig. 6J). To assess the functional significance of this upregulation, we depleted Myc from glial cells using RNAi. Loss of Myc severely reduced glial cell numbers in otherwise wild-type animals (Fig. 6D,N), and also suppressed the glial overgrowth phenotypes of both activated-Yki and ban (Fig. 6E,F). Thus, Yki- and ban-induced glial growth are Myc-dependent. On its own, Myc over-expression induces only a very mild glial overgrowth phenotype (Fig. 2H, Fig. 6G,O) (Read et al., 2009), but co-expression of Myc and Yki resulted in a much stronger glial over-proliferation phenotype than either gene alone (Fig. 6H,P), and was associated with substantial EdU-labeling of cortex glia even at late third instar, whereas expression of Myc or activated-Yki alone did not have this effect (supplementary material Fig. S1). Thus, Myc is a downstream target of Yki in glia, but Myc also acts synergistically with other Yki targets to induce glial cell proliferation.

**DISCUSSION**

**Regulation of glial growth by a Merlin-Hippo signaling pathway**

Merlin was first identified as the product of a human tumor suppressor gene, NF2, loss of which in peripheral glial cells results in benign tumors (Gusella et al., 1996). Merlin has also been identified as an inhibitor of gliomas (Lau et al., 2008). Our observations indicate that the role of Merlin as a negative regulator of glial cell proliferation is conserved from humans to *Drosophila* and, thus, that *Drosophila* can serve as a model for understanding Merlin-dependent regulation of glial growth.

Studies in *Drosophila* imaginal discs first linked Merlin to Hippo signaling (Hamaratoglu et al., 2006), and Merlin was subsequently linked to Hippo signaling in mammalian cells (Striedinger et al., 2008; Zhang et al., 2010), including its role in meningioma (Striedinger et al., 2008). However, the tumor suppressor activity of Merlin has also been linked to other downstream effectors in mammals, including Erb2, Src, ras, rac, TORC1 (CRT1 – Human Gene Nomenclature Database) and CRL4 (IL17RB – Human Gene Nomenclature Database) (Tikoo et al., 1994; Shaw et al., 2001; Curto et al., 2007; Morrison et al., 2007; Houshmandi et al., 2009; Lopez-Lago et al., 2009; Benhamouche et al., 2010; Li et al., 2010), creating some uncertainty regarding the general importance of the linkage of Merlin to Hippo in growth control. We found that depletion of *Merlin*, depletion of other tumor suppressors in the Hippo pathway, or expression of an activated form of Yki, all result in similar glial overgrowth phenotypes. Moreover, depletion of *Merlin* increased nuclear localization of Yki, and depletion of Yki suppressed the overgrowth phenotype of Merlin. Together, these observations clearly establish that the glial overgrowth phenotype associated with Merlin depletion in *Drosophila* is mediated through the Hippo signaling pathway.

A noteworthy feature of Hippo signaling in *Drosophila* glial cells is that Merlin appears to be uniquely required as an upstream regulator of Hippo signaling, as the Fat-dependent, Ex-dependent and Lgl-dependent branches have no detectable role. Glia might, thus, provide an ideal model for mechanistic investigations of the Merlin branch of Hippo signaling. Fat-Hippo signaling employs Fat as a transmembrane receptor and Dachsous as its transmembrane ligand (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006; Rogulja et al., 2008; Feng and Irvine, 2009), whereas Ex-Hippo signaling appears to employ Crumbs as a transmembrane receptor and ligand (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010). By contrast, *Drosophila* transmembrane proteins that mediate extracellular signaling and interact with Merlin have not yet been identified. Distinct mechanisms might also be involved in signal transduction downstream of Merlin. Although there is evidence that Ex and Merlin can both influence Hippo activity, Ex, but not Mer, can directly associate with Hpo. Conversely, Merlin, but not Ex, can interact directly with Salvador, and Merlin, Salvador and Hippo can form a trimeric complex. Moreover, the *kibra* loss-of-function phenotype is weaker than *expanded* in imaginal discs, but comparable to Merlin (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010), and we found that depletion of *kibra* also has a significant effect on glial cell proliferation. Kibra is highly expressed in mammalian brain, and alleles of *KIBRA* (*WWC1* – Human Gene Nomenclature Database) have been linked to human memory performance (Papassotiriopoulos et al., 2006). The role of *kibra* in regulating glial cell numbers in *Drosophila* thus raise the possibility that the influence of *KIBRA* on human memory might reflect a role in glial cells.

Finally, we note that although Hippo signaling has been investigated in several different organs in *Drosophila*, including imaginal discs, ovarian follicle cells, neuroepithelial cells and intestinal cells, these all involve roles in epithelial cells, in which upstream regulators of the pathway (e.g. Fat, Ex, Mer) all have a distinctive localization near adherens junctions. The identification of a requirement for Hippo signaling in glia is the first time in *Drosophila* that a role for the pathway has been identified in non-epithelial cells. Indeed, in previous studies we found that Hippo signaling influences proliferation of neuroepithelial cells, but other neuronal cell types, including neuroblasts, ganglion mother cells and neurons, are insensitive to Yki (Reddy et al., 2010).

**Identification of genes that promote glial growth**

Considerable attention has been paid to genes for which mutation or inappropriate activation can cause over-proliferation of glial cells, resulting in glial tumors. However, less is known about the mechanisms required for normal glial growth. Through loss-of-function studies, we identified several genes essential for normal glial cell numbers, including *yki, sd, ban, mad* and *myc*. The requirement for *yki, mad* and *sd*, together with epistasis studies, identifies a requirement for active Yki in glial growth. This in turn implies that downregulation of Hippo signaling is important for normal glial growth. Understanding how this is achieved will provide further insights into the regulation of glial cell numbers.

A requirement for Mad, together with its upstream regulator Thickveins (Tkv), in promoting retinal glial cell proliferation was described previously by Rangarajan et al. (Rangarajan et al., 2001). Our studies of glial cells, together with recent work in imaginal discs (Oh and Irvine, 2011), emphasize that in mediating the growth-regulating activity of Hippo signaling, Yki utilizes multiple DNA-binding partners (i.e. Mad and Sd) in the same cells at the same time to regulate distinct downstream target genes required for tissue growth.

Although Yki activity influenced glial cell numbers throughout the nervous system, direct analysis of cell proliferation by EdU labeling revealed that retinal glia were more sensitive to Yki activation at late third instar than central brain glia, and significant
induction of central brain glial cell proliferation was only observed when Yki activation was combined with Myc over-expression. Further studies will be required to define the basis for this differential sensitivity, but the implication that the proliferative response to Yki is modulated by developmental stage and/or glial cell type has important implications for diseases associated with both excess and deficits of glial cells.

A regulatory pathway for glial growth

Our studies in *Drosophila* delineate functional relationships among genes involved in the control of glial cell proliferation. Mammalian homologs of Merlin, Yki and Myc have been implicated in glioma (Herms et al., 1999; Uppal and Coatesworth, 2003; Lau et al., 2008; Striedinger et al., 2008). Although a mammalian homolog of ban has not been described, other miRNAs have also been linked to glioma (Silber et al., 2009). Our observations imply that these genes can be placed into a pathway, in which Merlin, through Hippo signaling, regulates Yki, Yki regulates ban, and ban regulates Myc. However, as expression of Myc alone did not lead to substantial overgrowth of glia, Yki and ban must also have other downstream targets important for the promotion of glial cell proliferation. Moreover, our observations indicate that a Yki-Sd complex is also required for glial growth. In addition to the well characterized downstream target Diap1, Yki-Sd complexes in glial cells might regulate Myc directly, as suggested by studies in imaginal discs (Neto-Silva et al., 2010), and might regulate cell cycle genes in conjunction with E2F1 (E2F–FlyBase) (Nicolaie et al., 2011).

The influence of activated-Yki on a *ban-GFP* sensor, together with the observations that *yki* is not required for ban-mediated overgrowth, whereas ban is required for Yki-mediated overgrowth, position ban downstream of Yki. This is consistent with studies of Hippo signaling in imaginal discs, in which ban has also been identified as a target of Yki for growth regulation (Nolo et al., 2006; Thompson and Cohen, 2006). The placement of Myc downstream of Yki and ban is supported by the observation that Myc levels can be increased by expression of ban or activated-Yki, and by genetic tests that indicate that Myc is required for Yki- and ban-promoted glial overgrowth. A mechanism by which ban can regulate Myc levels, involving downregulation of a ubiquitin ligase that negatively regulates Myc, was identified recently in imaginal discs (Herranz et al., 2010), and might also function in glial cells. Myc has been reported to downregulate Yki expression in imaginal discs (Neto-Silva et al., 2010) and, although we have not investigated whether a similar negative-feedback loop exists in glial cells, the synergistic enhancement of glial cell proliferation observed when Yki and Myc were co-expressed is consistent with this possibility, as the expression of both genes under heterologous promoters could bypass negative regulation of Yki by Myc.

The *Myc* proto-oncogene is de-regulated or amplified in several human cancers, including gliomas (Herms et al., 1999; Herms et al., 2000; Pellengris et al., 2002). The sensitivity of Yki/ban-induced overgrowth to reduced Myc levels parallels studies of glioma pathways and their implications for glioma therapy. Expert Rev. Anticancer Ther. 9, 235-245.


Merlin-Hippo signaling in glia


