Influence of Fat-Hippo and Notch signaling on the proliferation and differentiation of *Drosophila* optic neuroepithelia

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**SUMMARY**

The *Drosophila* optic lobe develops from neuroepithelial cells, which function as symmetrically dividing neural progenitors. We describe here a role for the Fat-Hippo pathway in controlling the growth and differentiation of *Drosophila* optic neuroepithelia. Mutation of tumor suppressor genes within the pathway, or expression of activated Yorkie, promotes overgrowth of neuroepithelial cells and delays or blocks their differentiation; mutation of *yorkie* inhibits growth and accelerates differentiation. Neuroblasts and other neural cells, by contrast, appear unaffected by Yorkie activation. Neuroepithelial cells undergo a cell cycle arrest before converting to neuroblasts; this cell cycle arrest is regulated by Fat-Hippo signaling. Combinations of cell cycle regulators, including E2f1 and CyclinD, delay neuroepithelial differentiation, and Fat-Hippo signaling delays differentiation in part through E2f1. We also characterize roles for Jak-Stat and Notch signaling. Our studies establish that the progression of neuroepithelial cells to neuroblasts is regulated by Notch signaling, and suggest a model in which Fat-Hippo and Jak-Stat signaling influence differentiation by their acceleration of cell cycle progression and consequent impairment of Delta accumulation, thereby modulating Notch signaling. This characterization of Fat-Hippo signaling in neuroepithelial growth and differentiation also provides insights into the potential roles of Yes-associated protein in vertebrate neural development and medulloblastoma.

**KEY WORDS:** Fat, Hippo, Neuroepithelia, YAP, Yorkie, Tumor

**INTRODUCTION**

Both normal development and homeostasis require that cells transition from proliferating undifferentiated cells to quiescent differentiated cells. Failure to undergo this transition results in tumor formation, whereas premature differentiation results in hypotrophy. Some tissues balance proliferation and differentiation by employing stem cells that divide asymmetrically to yield both a stem cell and a progenitor cell, which will then give rise to differentiated cells. Most of the *Drosophila* central nervous system develops in this way: individual cells within the embryonic ectoderm become specified as neural stem cells called neuroblasts (NBs), which divide asymmetrically to yield a neuroblast and a progenitor cell called a ganglion mother cell (GMC) (for a review, see Doe, 2008). By contrast, much of the vertebrate central nervous system initially develops from neuroepithelia (NE), sheets of epithelial neural progenitor cells that function as symmetrically dividing neural stem cells (for reviews, see Farkas and Huttner, 2008; Morrison and Kimble, 2006). This provides for rapid expansion of neural tissue, and then, as development proceeds, asymmetrically dividing progenitor cells arise, although the mechanisms that govern their appearance are not well understood. The optic lobe of *Drosophila* is unlike the rest of the *Drosophila* nervous system in that, akin to the vertebrate nervous system, it develops from NE (Egger et al., 2007; Hofbauer and Campos-Ortega, 1990). The optic lobe may thus serve as a model in which the powerful experimental approaches available in *Drosophila* can be used to investigate mechanisms that control the growth and differentiation of NE.

At the end of larval development, the optic lobes comprise the lateral half of each of the two brain hemispheres, and are organized into lamina, medulla and lobula layers (for a review, see Fischbach and Hiesinger, 2008). The optic lobes originate from clusters of epithelial cells that invaginate from a small region on the surface of the embryo (the optic placode) (Green et al., 1993). During larval development, these cells separate into an inner optic anlagen (IOA), which will give rise to the lobula and inner part of the medulla, and an outer optic anlagen (OOA), which will give rise to the outer part of the medulla and the lamina (Fig. 1A,D). Initially, the IOA and OOA are composed entirely of NE cells, but during the third larval instar they begin to differentiate. Along the lateral margin of the OOA, NE cells undergo cell cycle arrest in G1, and then are recruited to differentiate into lamina neurons by signals from the arriving retinal axons (for a review, see Kunes, 2000). Along the medial margin of the OOA, a wave of differentiation sweeps across the NE from medial to lateral, converting NE cells into medulla NBs (Fig. 1B,C) (Egger et al., 2007; Yasugi et al., 2008). These NBs divide perpendicularly to the plane of the neuroepithelium, and appear to follow a NB developmental program, giving rise to additional self-renewing NBs, and to GMCs, which ultimately give rise to neurons.

The Fat-Hippo signaling pathway encompasses distinct downstream branches that regulate planar cell polarity and gene expression (for a review, see Reddy and Irvine, 2008) (Fig. 1E). Transcriptional targets of the pathway include genes that influence cell proliferation and cell survival, and consequently Fat-Hippo signaling is an important regulator of growth from neuroepithelia to vertebrates. The influence of Fat-Hippo signaling on transcription...
is mediated by a co-activator protein, called Yorkie (Yki) in *Drosophila* and Yes-associated protein (YAP) in vertebrates (Huang et al., 2005). Warts (Wts)-mediated phosphorylation and binding to cytoplasmic proteins negatively regulate Yki by promoting its retention in the cytoplasm (Badouel et al., 2009; Dong et al., 2007; Oh and Irvine, 2008; Oh et al., 2009; Zhao et al., 2007). Wts is regulated in at least two ways: Wts kinase activity is promoted by Hippo; and Wts protein levels are influenced by Dachs (for a review, see Reddy and Irvine, 2008). Upstream regulators of the pathway include the large cadherin Fat, and the FERM-domain proteins Merlin (Mer) and Expanded (Ex) (Bennett and Harvey, 2006; Feng and Irvine, 2007; Silva et al., 2006; Willecke et al., 2006; Zhao et al., 2007). Genetic studies in *Drosophila* have also revealed that the relative contributions of pathway components can vary among different tissues (for a review, see Reddy and Irvine, 2008).

Optic NE cells proliferate during larval development, but aside from a requirement for the transcription factor DVSX1 (Erclik et al., 2008), how this proliferation is regulated is not understood. The progression of NE cells to medulla NBs in the OOA is antagonized by Jak-Stat signaling (Yasugi et al., 2008), but, aside from this, the regulation of this differentiation wave is not understood. Here, we demonstrate that Fat-Hippo signaling regulates the proliferation and differentiation of NE cells in the optic lobe. By contrast, Fat-Hippo signaling does not detectably influence the proliferation or differentiation of NBs or their progeny. We also identify a role for Notch signaling in controlling the progression of NE cells to medulla NBs, and characterize relationships between the Fat-Hippo, Jak-Stat and Notch signaling pathways. Our results indicate that a transient pause in the cell cycle is needed for cells to transition from NE cells to NBs, and suggest a model in which a cell cycle arrest modulates Notch signaling by contributing to accumulation of Delta expression. The insights our results provide into the role of Fat-Hippo signaling in NE growth and differentiation in *Drosophila* are likely to be relevant to recently described roles of YAP in vertebrate neural development and medulloblastoma (Cao et al., 2008; Fernandez et al., 2009).

**MATERIALS AND METHODS**

**Drosophila genetics**

Ectopic expression clones were created by Flip-out using:

- hs-Flip[122]; act>y'=gal-4 UAS-GFP,
- UAS-yki:V5,
- UAS-yki:V5<sup>1064</sup>,
- UAS-Yki-S168A;GFP[10-12-1],
- UAS-hop,
- UAS-dap,
- hs-Flip[122]; UAS-E2f1 UAS-Dp,
- hs-Flip[122]; UAS-CycE UAS-Cdk2,
- UAS-CycD UAS-Cdk4,
- act>y'=gal-4 UAS-LacZ,
- hs-Flip[122]; UAS-CycD UAS-Cdk4; UAS-E2f1 UAS-Dp,
- hs-Flip[122]; UAS-CycD UAS-Cdk4; UAS-CycE UAS-Cdk2,
- hs-Flip[122]; UAS-E2f1 UAS-Dp; UAS-CycE UAS-Cdk2,
- UAS-ΔN34a/Cyo and hs-Flip; 10Xstat GFP; UAS-yki:V5<sup>1064</sup>.

Some mutant clones were created by FLP-FRT-mediated recombination, using:

- hs-Flip[122]; Ubi-GFP FRT40A,
- w; ex<sup>1</sup> FRT40A/Cyo-GFP,
- Mer<sup>+</sup>; P[w=+/Ubi-Mer+] FRT40A/Cyo-GFP,
- hs-Flip: FRT42B Ubi-GFP;nsCyO,
- FRT42B dap<sup>+</sup>Cyo Act-GFP and FRT18A N<sup>53el</sup>.

**Mer ex double mutant clones were made using:**

- Mer<sup>+</sup>; P[w=+/Ubi-Mer+] FRT40A/Cyo-GFP,
- Mer<sup>+</sup>; Ubi-GFP P[w=+/Ubi-Mer+] FRT40A/Cyo-GFP and ex<sup>2</sup> FRT40A/Cyo-GFP;
- hs-FLP Sb/TM6b.

**yki<sup>+</sup>** Minute clones were made using FRT42D yki<sup>+</sup>Cyo and FRT42D M H1378U.

**Mutant clones positively marked with GFP were made using:**

- yki<sup>+</sup> FRT82B/TM6b,
- w; FRT82B Staur<sup>92E</sup> /TM6b,
- w; FRT82B Staur<sup>92E</sup>Cyo/TM6b,
- UAS-Yki;GFP; FRT82B yki<sup>+</sup>/TM6b,
- ex<sup>1</sup> FRT40A/Cyo-GFP.
Markers of gene expression and activity included 10XStat-Gfp washed with PBS and fixed with 4% paraformaldehyde in PBS. Flour Imaging kits (Invitrogen) with Alexa-594 azide. Dissected larvae and anti-L(1)sc (A. Carmena), pig anti-E2F1 (1:1000, D. Dimova), rabbit anti-Hth (1:400, A. Salzberg) phalloidin (1:100, Invitrogen), mouse anti-Dap (1:400, B. Nicolay), guinea pig anti-E2F1 (1:1000, D. Dimova), rabbit anti-Hth (1:400, A. Salzberg) and anti-L(1)sc (A. Carmena).

EdU labeling and detection was performed using Click-iT EdU Alexa Flour Imaging kits (Invitrogen) with Alexa-594 azide. Dissected larvae were incubated with EdU (20 μM) at room temperature for 10 minutes, washed with PBS and fixed with 4% paraformaldehyde in PBS.

RESULTS
Most prior studies of Fat-Hippo signaling in Drosophila have focused on its role in regulating imaginal disc growth. To investigate Fat-Hippo signaling during neural development, we have examined the expression of pathway components. The larval central nervous system comprises the condensed ventral nerve cord (VNC) and two attached brain hemispheres (Fig. 1D). The medial half of each brain hemisphere develops from four mushroom body NBs and 85 central brain NBs (Ito and Hotta, 1992), whereas the lateral half comprises the optic lobe (Fig. 1B). Throughout the brain, the progression of development can be visualized using molecular markers, including Deadpan (Dpn) for NBs, Prospero (Pros) for GMCs, and Elav for neurons. The distinctive morphology of the optic NE can be revealed by staining for Armadillo (Arm), E-cadherin (E-cad) or F-actin (using phalloidin).

Expression of Fat-Hippo pathway components in the brain
Fat and Ex are expressed specifically throughout optic NE cells in the larval brain, and localized to the subapical membrane, similar to their localization within imaginal disc cells (Fig. 2A,B) (Fung et al., 2008). The Fat pathway modulator lowfat is also expressed in NE (Mao et al., 2009). Mer is expressed at similar levels in the NE and throughout the rest of the brain (Fig. 2C). The Fat regulators Ds and Fj are expressed within the NE (Fig. 2D,E) (Fung et al., 2008), and, as in imaginal discs, they are expressed in complementary gradients. Ds expression is highest at the two ends of the U-shaped optic NE (Fig. 2D). Available antibodies against Fj did not detect expression in situ, but using a fj-lacZ reporter, fj expression was highest in the center of the NE (Fig. 2E). In imaginal tissues, gradients of fj and ds expression can influence both the Warts-Hippo and PCP branches of Fat signaling (reviewed by Reddy and Irvine, 2008), and the most direct response to these gradients is a polarized localization of Dachs, which can be visualized by expression of a tagged form: Dachs:V5 (Mao et al., 2006). Dachs:V5 localization was also polarized in the optic NE, and as in imaginal discs was detected at the membrane along the sides of cells closest to peak fj expression and farthest from peak Ds expression (Fig. 2F). This implies that Fj and Ds signal through Fat to influence Dachs in the optic NE.

Although there are multiple upstream inputs into Fat-Hippo signaling, the influence of this pathway on growth all appears to be mediated by regulation of Yki. The expression of Yki is also spatially restricted in the brain. Strong Yki expression was detected throughout the NE, but Yki was not detectably expressed in the differentiating NBs or lamina neurons (Fig. 2G-I). Thus, there must exist a mechanism for repressing Yki expression as NE cells differentiate. Yki was predominantly cytoplasmic in NE cells, with faint staining detectable in the nucleus, similar to its localization in imaginal discs (Dong et al., 2007; Oh and Irvine, 2008). Most of the central brain did not express Yki, except for cells that, based on their shape, location and expression of repo-Gal4, were identified as glial cells. The Yki co-factors Scalloped (Sd) and Homothorax (Hth) (Peng et al., 2009; Wu et al., 2008; Zhang et al., 2008) were expressed both in the NE and in other regions (see Fig. S1 in the supplementary material).

Fat-Hippo signaling functions within the NE and regulates growth
In imaginal discs, mutation of upstream tumor suppressors of the pathway results in overgrowth, and their effects on pathway activity can be visualized by increased nuclear Yki (Dong et al., 2007; Oh and Irvine, 2008). We examined clones of cells mutant for wt, Mer or ex, or doubly mutant for Mer ex, dachs fat or fat ex, in the optic NE. In all cases except for Mer and dachs fat, mutant clones grew obviously larger than control clones, indicative of a more rapid growth rate. They were also rounder and had smoother borders than control clones, suggesting differences in adhesion, as has been observed in imaginal discs. Additionally, they were associated with increased nuclear Yki (Fig. 3; see Fig. S2 in the supplementary material). The strongest effects on Yki localization and growth were observed in wts mutant clones and in Mer ex or fat ex double mutant clones, whereas single mutant fat or ex mutant clones tended to have weaker effects, similar to their relative influence in imaginal discs (Oh and Irvine, 2008). Genetically, dachs is required for the effects of fat on gene expression and growth in imaginal discs (Cho et al., 2006; Cho and Irvine, 2004; Mao et al., 2006), and mutation of dachs similarly suppressed the effects of fat on Yki localization and growth in NE clones (Fig. 3E). Together, these observations indicate that each of the three most upstream autonomous regulators of Fat-Hippo signaling in imaginal discs (i.e. Fat, Ex and Mer) contribute to the regulation of Fat-Hippo signaling in the optic NE.

To assess directly the influence of Yki activation on NE cells, we took advantage of the observation that mutation of a conserved Wts phosphorylation site within Yki, at Ser168, hyperactivates Yki (Dong et al., 2007; Oh and Irvine, 2008). Expression of Yki:V5S168A in imaginal discs under UAS-Gal4 control promotes strong over-growth, whereas expression of wild-type Yki:V5 at the same levels has no effect (Oh and Irvine, 2009). Similarly, expression of Yki:V5S168A, but not of wild-type Yki:V5, promoted overgrowth of optic NE (Fig. 4; see Fig. S3 in the supplementary material). Expression of Yki:V5S168A in clones of cells in the NE resulted in large round clones with smooth borders (Fig. 4B,E-L). The overgrowth of these clones was more extreme than in the upstream tumor suppressor mutants. When measured only 48 hours after clone induction, Yki:V5S168A NE clones were, on average, more than twice as large as control clones, and also exhibited increased labeling for phospho-histone H3 (pH3), indicative of faster proliferation (see Fig. S3F-I in the supplementary material). We
also examined the consequences of general activation of Yki throughout the NE by expressing Yki:V5S168A under the control of either arm-Gal4, which drives broad but low level expression, especially in epithelial tissues, and C855a-Gal4, which drives expression within the NE cells of the optic lobe (Egger et al., 2007). In both cases, enlarged brains were observed, with massively expanded and highly folded NE (Fig. 4G,I). We focus here on the larger and more accessible OOA, but note that Yki activation also affects the IOA (Fig. 4G; see Fig. S3B in the supplementary material). Overgrowth and folding of the NE was also observed in fat or ex mutant larvae (Fig. 4J,K), although not as severe as that induced by activated Yki. We also confirmed that a direct target of Yki, Drosophila Inhibitor of apoptosis protein 1 (Diap1) (Wu et al., 2008; Zhang et al., 2008), was upregulated in clones of NE cells expressing Yki:V5S168A (Fig. 4L). In contrast to the overgrowth of activated-Yki clones, clones mutant for yki grew poorly (see Fig. S3J in the supplementary material), even when the Minute technique was used to give them a growth advantage (Fig. 5E), which indicates that yki is required for the normal proliferation of NE cells.
Activation of Yki blocks differentiation of neuroepithelial cells but not neuroblasts

Yki:V5S168A-expressing clones within the optic lobe maintained epithelial morphology, exhibiting characteristic expression of markers of NE cells (Arm, E-cad, F-actin) and failing to express markers of more differentiated cells (Dpn, Pros, Elav, Dac; Fig. 4B-E). These clones were always rounded and overgrown compared with control clones, becoming folded as they increased in size (Fig. 4; see Fig. S3 in the supplementary material). In some cases, the overgrowth was so extreme that clones extended well into the central brain, but because at least part of the clone was always found within the optic lobe, we infer that they originated from the optic NE. These observations indicate that expression of Yki:V5S168A blocks the differentiation of optic NE cells into medulla NBs. Yki activation also inhibits differentiation into lamina neurons along the lateral side of the NE (Fig. 4E).

By contrast, Yki:V5S168A-expressing clones induced within the central brain expressed markers of more differentiated cells, and in most cases included only a single NB, indicating that they did not impair the progression of NBs to more differentiated cells (the occasional presence of multiple NBs was consistent with the juxtaposition of independently generated clones) (Fig. 4B; see Fig. S3 in the supplementary material). Although we did not analyze specific NB lineages, overall central brain clones appeared similar in size and shape to control clones, and Yki:V5S168A also did not appear to affect medulla neuroblasts (see Fig. S3 in the supplementary material). These observations suggest that NBs and their more differentiated progeny within the brain are refractory to the oncogenic effects normally associated with Yki activation.

Mutation of Fat-Hippo pathway tumor suppressors delays medulla neuroblast differentiation

In contrast to the complete block to NE differentiation observed in Yki:V5S168A-expressing clones, cells within NE clones mutant for upstream tumor suppressors, including wts, fat, ex, ex fat or Mer ex, were able to differentiate into medulla NBs, but their differentiation was delayed (Fig. 5; see Fig. S2 in the supplementary material). The differentiation wave normally sweeps evenly across the NE from medial to lateral (Yasugi et al., 2008), and the delay in differentiation was evident as cells within mutant clones retained their epithelial character, whereas neighboring wild-type cells had already become NBs. This was a delay in differentiation, and not simply a distortion caused by overgrowth within the NE, because even small clones that only barely touched the NE could cause a delay (Fig. 5C). The earliest known marker of the differentiation wave is the expression of the proneural protein Lethal(1)scute [L(1)sc], which becomes expressed in cells at the edge of the NE just before they differentiate into NBs, and promotes their progression to NBs (Fig. 6; see Fig. S4 in the supplementary material) (Yasugi et al., 2008). The delay in differentiation associated with mutation of tumor suppressors in the Fat-Hippo pathway was evident when L(1)sc expression was examined (Fig. 5C).

In wild type there is a complementarity between the Yki-expressing NE cells and the Dpn-expressing NBs (Fig. 2G). This complementarity was maintained within clones of cells mutant for Fat-Hippo pathway tumor suppressors (see Fig. S2 in the supplementary material). Thus, expression of Yki persisted as long as the cells maintain their NE character, but was lost as cells started to upregulate Dpn. We hypothesized that the weaker phenotype of tumor suppressor mutant clones, when compared with
Yki:V5^{S168A}-expressing clones, could indicate that expression of endogenous Yki is limiting. This was confirmed by the observation that clones of cells mutant for \textit{wts}, but simultaneously overexpressing wild-type Yki:V5 (which has no phenotype on its own), exhibited a complete block in NE differentiation (Fig. 4M), equivalent to the phenotype of Yki:V5^{S168A}-expressing clones. In complementary experiments, we observed that expression of a weak activated-Yki transgene (UAS-Yki-S168A:GFP\[10-12-1]\) (Oh and Irvine, 2008) only delayed differentiation, rather than blocking it (not shown).

By contrast to their obvious influence on the NE, \textit{fat}, \textit{wts}, \textit{ex}, \textit{ex} \textit{fat} or \textit{Mer} \textit{ex} clones did not have obvious effects on the development of the central brain, and, as described above for Yki:V5^{S168A}-expressing clones, mutant clones could be identified that appeared to be of normal size and expressed the normal complement of differentiating cell types (Fig. 5; see Fig. S2 in the supplementary material).

To determine whether Yki was not only sufficient to inhibit NE differentiation when activated, but also necessary for the normal timing of differentiation, we examined \textit{yki} mutant clones. These exhibited a slight acceleration of differentiation, as visualized by staining for expression of \textit{L(1)sc} and Dpn (Fig. 5E). Although mutation of \textit{yki} could accelerate the differentiation wave, mutant clones completely within the NE did not exhibit any signs of premature differentiation. Thus, Yki is not required autonomously to inhibit NE differentiation, but rather influences events at the differentiation wave.

**Relationship between Fat-Hippo and Jak-Stat signaling**

The delay in medulla NB differentiation associated with mutation of \textit{wts} or other tumor suppressors in the Fat-Hippo pathway is reminiscent of the differentiation delay associated with activation of Jak-Stat signaling (Yasugi et al., 2008).
differentiation induced by mutation of supplementary material). This was in contrast to the accelerated delayed NB differentiation (Fig. 6D; see Fig. S4D in the supplementary material). These observations imply that these two pathways can act in parallel to influence NE to NB progression. Altogether, our results suggest that the influence of Fat-Hippo signaling on differentiation is mediated at least in part through its effects on DI; DI upregulation is also delayed within Hop-expressing clones (see Fig. S4F in the supplementary material).

**Relationship between Fat-Hippo and Notch signaling**

Neurogenesis, and the expression of proneural genes, are negatively regulated by Notch in many contexts. Expression of the Notch ligand Delta (Dl) is upregulated along the medial edge of the NE, overlapping L(1)sc expression (Fig. 6E,F). Moreover, expression of activated Notch prevented both L(1)sc expression (Fig. 6H) and the NE to NB transition (Fig. 6G), whereas mutation of Notch accelerated NB formation (Fig. 6Q). This was not due to an influence on Fat-Hippo signaling, because Yki expression and localization were unaffected (Fig. 6I). Activation of Yki also repressed L(1)sc expression (Fig. 6J), but whereas activated Yki and activated Notch have similar effects on L(1)sc, they have opposite effects on DI: activated Notch promoted DI expression (Fig. 6M), and activated Yki repressed it (Fig. 6K). Upregulation of DI expression within wts mutant clones was not completely blocked, but instead was delayed (Fig. 6L). DI has complex effects on Notch: it acts non-autonomously as a ligand that activates Notch on neighboring cells, but it also acts cell-autonomously to prevent Notch within the same cell from being activated by ligands expressed by neighboring cells (for a review, see D’Souza et al., 2008). Consistent with the dual role of DI in Notch regulation, clones of cells mutant for DI exhibited complex effects, including both inhibition and acceleration of NB formation (Fig. 6N-P), which together with the effects of Notch, implicate Notch signaling as a regulator of NE to NB progression. Overexpression of Delta also had complex effects, both accelerating (Fig. 6V) and inhibiting (not shown) NB formation. Altogether, our results suggest that the influence of Fat-Hippo signaling on differentiation is mediated at least in part through its effects on DI; DI upregulation is also delayed within Hop-expressing clones (see Fig. S4F in the supplementary material).

**Cell cycle regulation in the optic lobe**

Many tissues undergo a transition from proliferating undifferentiated progenitor cells to quiescent differentiated cells. However, the transition from optic NE to NB is not associated with quiescence or terminal differentiation, because NBs continue to proliferate. Indeed, labeling of S phase cells with EdU implies that optic lobe NBs proliferate more extensively than NE cells (Fig. 7A). EdU labeling also revealed that cell proliferation in NE is heterogeneous, with groups of contiguous cells labeled, and other groups of cells unlabeled. Notably, cells along both the lamina and medulla edge of the outer NE were almost always unlabeled (Fig. 7A), which implies that they have undergone a cell cycle arrest. Cell cycle arrest along the lamina side of the NE has been investigated previously, and is reversed by signaling from retinal axons (reviewed in Kunes, 2000). The arrest along the medulla side has not been characterized. This cell cycle arrest was also reflected in the reduced expression of a marker of cell cycle progression: PCNA-GFP (Fig. 7B). Owing to the curved shape of the optic lobe, this was most visible in cross-section (Fig. 7C). The upregulation of DI and L(1)sc expression at the edge of the NE overlaps the region of cell cycle arrest (Fig. 6R,S).

To investigate whether this cell cycle pause along the medial edge of the NE was influenced by Fat-Hippo signaling, we performed EdU labeling on brains with clones of cells mutant for wts or expressing Yki;V5S168A. These clones prevented the normal cell cycle arrest at the edges of the NE, and were associated with EdU labeling that was more extensive than in wild-type NE cells, though reduced compared with wild-type optic lobe NBs (Fig. 7D,E). Thus, Fat-Hippo signaling regulates cell cycle progression.
in the optic lobe, and impairing Fat-Hippo signaling can prevent
the normal cell-cycle arrest. The cell cycle arrest was also delayed
by overexpression of Hop (see Fig. S4G in the supplementary
material). Activation of Notch, by contrast, reduced EdU labeling
in the NE (see Fig. S4H in the supplementary material).

These observations suggest a model in which cells need to
undergo a transient arrest in the cell cycle in order to progress from
NE to NBs. This model predicts that expressing proteins that
promote cell cycle progression could delay differentiation. To test
this, we expressed: E2f1 together with its co-factor Dp; CycE and
its associated kinase Cdk2; or CycD and its associated kinase
Cdk4. A slight delay in differentiation was observed in 8/26
CycD+Cdk4-expressing clones, whereas E2f1+Dp or CycE+Cdk2-
expressing clones had no effect (Fig. 7F,G; see Fig. S5D in the
supplementary material). Studies of a cell cycle pause associated
with retinal differentiation in the eye imaginal disc (Firth and
Baker, 2005), and of terminal differentiation of wing disc cells
(Butitta et al., 2007), have found that simultaneous manipulation
of multiple regulators is required to override normal cell cycle
control. Co-expression of E2F1+Dp with CycE+Cdk2, or
CycE+Cdk2 with CycD+Cdk4, did not result in additive
phenotypes (see Fig. S5E,F in the supplementary material).

Fig. 6. See next page for legend.
Optic neuroepithelial development

DEVELOPMENT

Fig. 6. Relationship of Fat-Hippo to Jak-Stat and Notch signaling.

Regions of larval brains, centered on the NE to NB transition. Panels marked by prime show stains without the clone marker, selected clones are identified by dashed outlines or arrows. In A-D, G-O, clones are marked by GFP (blue/green); brains are stained for Dpn (green) in A-D, G-H, I, N, O, Q; Yki (red) in A, B, I; E-cad (red/blue) in C-D, K-O, Q; Dl (red/green) in E, F, K-L; L(1)sc (red) in F, H, J. (A) Clones expressing hop under act-Gal4 control. (B) Clones co-expressing hop and yki RNAi, the effectiveness of RNAi is indicated by reduced Yki. (C) Stat92E(wts1) mutant clones accelerate differentiation. (D) Stat92E(wts1) mutant clones delay differentiation. (E, F) DI expression at the edge of the NE in wild type. (G-H) Clones expressing activated Notch block differentiation (G), block L(1)sc expression (H), but do not influence Yki localization (I). (J-K) Clones expressing activated Yki block L(1)sc expression (J) and downregulate DI expression (K). (L) wts mutant clones delay DI expression. (M) Clones expressing activated Notch upregulate DI. (N-P) Clones mutant for Dpr11p. (Q) Apical section in which both autonomous delay and non-autonomous acceleration of differentiation are visible. (O) Basal section of the same sample, autonomous acceleration of differentiation is visible; these premature NBs often drop basally. (P) Schematic interpretation of Delta expression and the consequences of a Dl mutant clone. In the interior of a clone, Notch activity is lost (1). We propose that these cells become NBs and drop basally (asterisk in O). Around the edges of a clone (2), autonomous inhibition of Notch activity is lost but Notch can be activated by Delta from neighboring cells. Notch activity therefore increases, delaying NB formation (yellow arrow in N). Outside the edges of a clone (3), Notch activity is reduced owing to decreased DI signaling from neighboring cells, accelerating NB formation (white arrow in N). (Q) Accelerated NB formation in a Notch mutant clone. (R, S) Expression of DI (R) and L(1)sc (S) at the edge of the NE is detected in wild-type cells that do not label with EdU (red). (T-U) Clones expressing CycD+Cdk4+E2f1+Dp do not directly affect Yki (T) or Fat (U) expression, but expression of Yki and Fat is maintained as long as cells remain NE. (V) Projection through apical and basal regions of a clone over-expressing DI. Some cells within DI-expressing clones become NBs and drop basally (arrow).

However, when E2F1+Dp were co-expressed with CycD+Cdk4, 17/32 clones delayed the progression of NE into NBs, and the delay was more extensive and observed in smaller clones than for expression of CycD+Cdk4 alone (Fig. 7I). Thus, progression of NE into NBs can be modulated by cell cycle regulators. When a differentiation delay was induced by co-expression of E2F1, Dp, CycD and Cdk4, there was a corresponding delay in the upregulation of DI expression (Fig. 7J). Expression of Yki and Fat was maintained as long as cells maintained NE character (Fig. 6T,U). PCNA is a direct target of E2f1 (Thacker et al., 2003); hence, the reduced PCNA-GFP expression at the edges of the NE (Fig. 7B,C) implies that E2f1 activity is low, even though E2f1 itself was expressed throughout the neuroepithelium (see Fig. S6A in the supplementary material). Expression of Dacapo (Dap), a Drosophila cyclin-dependent kinase inhibitor, was elevated at the edge of the NE (see Fig. S6B in the supplementary material), and so might contribute to cell cycle arrest. However, neither mutation nor overexpression of Dap had visible effects on differentiation (see Fig. S5A-C in the supplementary material), suggesting that its contribution is redundant with other factors. Fat-Hippo signaling regulates E2f1 expression in imaginal discs (Goulev et al., 2008; Nicolay and Frolov, 2008), and increases in both e2f1-lacZ and CycE-lacZ could be observed in Yki;V5168A-expressing clones in the NE (see Fig. S6D,E in the supplementary material). In functional tests, mutation of e2f1 suppressed the wts-mediated differentiation delay (Fig. 7J; 100% of wts mutant clones delay differentiation, but only 12/29 wts E2f1 double mutant clones delayed differentiation; the delays observed were quite modest compared with those in wts mutant clones). These observations implicate E2f1 as a regulator of NE to NB progression, presumably owing to its influence on cell cycle progression.

DISCUSSION

The Fat-Hippo pathway has emerged as an important regulator of growth, but has not previously been implicated in neural development in Drosophila. The observation that expression of an activated form of Yki, or mutation of tumor suppressors in the pathway (i.e. fat, ex or wts), promotes growth, whereas mutation of yki impairs growth, identify a crucial role for Fat-Hippo signaling in regulating the proliferation of optic neural progenitor cells (i.e. NE). Indeed, expression of activated Yki can result in massive overgrowths that are taken up in folded sheets of NE, which push into the central brain, forming tumors of undifferentiated NE cells. Although the influence of Fat-Hippo signaling on NE growth parallels its influence on imaginal discs, the influence of Fat-Hippo signaling on NE differentiation (see Fig. S7 in the supplementary material) does not, as clones of cells mutant for tumor suppressors in the pathway can differentiate cuticle in the head, thorax and abdomen.

In contrast to the extensive overgrowth and suppressed differentiation of NE, NBs and their more differentiated progeny appear refractory to Fat-Hippo signaling. Developing tissues that are unaffected by Fat-Hippo signaling have not been well characterized. The restriction of Fat-Hippo signaling to the NE is matched by the preferential expression of several pathway components, but even when a constitutively activated form of Yki was expressed outside of the NE, neural development in the central brain was not obviously perturbed. Given the emerging importance of Hippo signaling in cancer, determination of what makes different cell types sensitive or resistant to activated Yki is an important direction for future studies.

The progressive nature of NE to NB differentiation in the optic lobe, with different stages displayed in a spatial pattern, make it a sensitive system for investigating differentiation. The extent of delay associated with Fat-Hippo pathway tumor suppressors varied depending on strength of the mutations, which suggests that progression of NE to NB involves a balance of positive and negative influences. The silencing of Yki expression as cells differentiate further suggests that there is negative feedback of differentiation signals onto Yki, which might normally help to ensure a sharp transition between NE and NBs. When Yki activity is further elevated, by overexpression of activated Yki, a complete block in differentiation could be achieved. The observation that a complete block in differentiation could also be achieved by combining overexpression of wild-type Yki with a mutation that influences Yki phosphorylation (wts) is intriguing in light of observations that several human cancers are associated with an increase in levels of Yki expression, rather than a simple change in its localization or phosphorylation (Fernandez et al., 2009; Overholzer et al., 2006; Steinhardt et al., 2008). Thus, we suggest that the two-hit scenario observed in the optic lobe, in which both Yki activity and Yki levels need to be affected in order to transform cells permanently, could also be relevant to human tumors.

Our analysis of optic lobe development and the influence of Fat-Hippo signaling implies that a transient pause in the cell cycle is required for cells to transition from NE to medulla NBs, and that
Fat-Hippo signaling influences differentiation via an effect on the cell cycle. This model is supported by several observations: there is normally a cell cycle pause along the edge of the OOA NE; inhibition of Fat-Hippo signaling, or activation of Yki, impairs both this cell cycle pause and differentiation; and direct manipulation of multiple cell cycle regulators can delay NE differentiation. Although multiple cell cycle regulators appear to be involved in this cell cycle pause, our analysis implicates E2f1 as a key player. PCNA-GFP is downregulated at the edge of the NE, which indicates that E2f1 activity is low there. As E2f1 activity is negatively regulated by association with Rb, and Rb is negatively regulated by phosphorylation by Cdns, expression of CycD+Cdk4 is expected to increase E2f1 activity. Thus, the significant delay in differentiation observed when CycD+Cdk4 were co-expressed with E2f1+DP could all be due to increased E2f1 activity. Importantly, E2f1 is normally regulated by Fat-Hippo signaling in the optic NE, and E2f1 is functionally important for the influence of Fat-Hippo signaling on NE differentiation, because mutation of E2f1 suppressed the wts-mediated differentiation delay. A cell cycle pause also occurs in conjunction with a wave of differentiation that sweeps across the developing eye imaginal disc; however, direct manipulation of cell cycle progression did not affect the...
differentiation wave in the eye disc (Firth and Baker, 2005), nor does mutation of wts, hpo or sav affect differentiation of photoreceptor cells, even though it does prevent the normal cell cycle pause in the eye disc (for a review, see Hariharan and Bilder, 2006).

The transition from NE to NB is regulated by Notch signaling, and our results suggest a model in which high level expression of DI at the edge of the NE autonomously inhibits Notch activation, resulting in upregulation of L(1)sc, which promotes NB fate (see Fig. S7 in the supplementary material). This model is supported by the observations that activation of Notch or mutation of DI can inhibit NE differentiation. At the same time, high-level expression of DI should enhance Notch activation in neighboring cells, which, as DI is upregulated by Notch activation, would contribute to the progressive spread of elevated DI expression across the NE. This simple model allows for the input of other pathways into NE to NB progression via effects on DI expression, and indeed this appears to be the point at which Fat-Hippo and Jak-Stat signaling intersect with Notch. As a unifying model (see Fig. S7C in the supplementary material), we propose that a cell cycle pause facilitates the accumulation of the high levels of DI expression needed to autonomously block Notch signaling, and thereby to upregulate the expression of proneural genes like L(1)sc. A possible mechanism for this hypothesized effect on Delta is suggested by the recent observation in vertebrate NE that Delta1 transcripts are unstable during S-phase (Cisneros et al., 2008). The hypothesis that the influence of Fat-Hippo signaling on differentiation is due to its effect on DI expression also provides an explanation for the specificity of this phenotype, as DI is not generally required for the differentiation of imaginal disc cells.

Studies of homologues of Yki, Sd, Hpo and Wts in the chick neural tube identified influences on proliferation and differentiation (Cao et al., 2008). These studies identified effects on Sox2-expressing neural progenitor cells, but could not distinguish between effects on NE cells versus other neural progenitor cells. A recent study has also implicated YAP in Hedgehog-associated medulloblastoma (Fernandez et al., 2009). Vertebrate NE cells give rise to progenitor cells (e.g. radial glial cells and basal progenitors) that share with neuroblasts the ability to divide asymmetrically to give rise to both another progenitor cell and a more differentiated cell. As our analysis of the Drosophila optic lobe indicates that Fat-Hippo signaling functions specifically to regulate the proliferation and differentiation of NE, we suggest that YAP might also function specifically within NE cells in vertebrates. Notably, the observation that depending on the level of expression, Yki can delay rather than block differentiation, provides for the possibility that YAP-dependent tumors could nonetheless contain a mixture of NE cells and more differentiated cells. In Drosophila, each of the three upstream branches of the pathway (i.e. Fat-dependent, Ex-dependent and Mer-dependent, Fig. 1E), contribute to Yki regulation in NE. Studies in vertebrates have not addressed how the pathway is normally regulated, but Fat-, Ds- and Fj-related genes are all normally expressed in vertebrate NE (Ashery-Padan et al., 1999; Rock et al., 2005; Yamaguchi et al., 2006), consistent with the possibility that they function there.

Artificially slowing the cell cycle can promote precocious differentiation in the cortex (Calegari and Huttner, 2003; Lange et al., 2009), although in this context increasing cell cycle length was associated with a transition from proliferative to differentiative divisions of basal progenitors, which appear functionally similar to NBs rather than to NE cells. The differentiation of optic lobe NE cells into medulla NBs also differs from the general model of increasing cell cycle length causing differentiation, because NBs proliferate even more rapidly than NE cells, and thus this step is not associated with a general lengthening of the cell cycle, but rather a transient pause. Nonetheless, it is intriguing that, in the spinal cord, overexpression of CyclinD did not block differentiation, but did appear to transiently delay it (Lobjois et al., 2008; Lobjois et al., 2004), reminiscent of the delay in NE to NB progression that we identified in the optic lobe. Moreover, CyclinD expression is regulated by Hippo signaling in the chick neural tube (Cao et al., 2008), and overexpression of CyclinD inhibits differentiation there. Although further studies are required to identify the CyclinD-sensitive mechanism in the vertebrate nervous system, the reported instability of Delta1 transcripts during S phase, together with the role of Notch signaling in maintaining NE progenitors in vertebrates (Henrique et al., 1997) and our analysis of NE differentiation and DI expression in the Drosophila optic lobe, suggest that the possibility of a general influence of cell cycle progression on Notch signaling warrants further investigation as a contributor to the link between cell cycle progression and differentiation in the nervous system across different phyla.

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