The Hippo pathway regulates apical-domain size independently of its growth-control function

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Summary

The Hippo pathway, identified in Drosophila and conserved in vertebrates, regulates tissue growth by promoting cell cycle exit and apoptosis. In addition to their well-characterised overproliferation phenotype, adult Drosophila epithelial cells mutant for the kinases Hippo and Warts have hypertrophic apical domains. Here we examine the molecular basis of this apical hypertrophy and its impact on cell proliferation. In the wing imaginal disc epithelium, we observe increased staining for members of the apical polarity complexes aPKC and Crumbs as well as adherens junction components when Hippo activity is compromised, while basolateral markers are not affected. This increase in apical proteins is correlated with a hypertrophy of the apical domain and adherens junctions. The cell surface localisation of the Notch receptor is also increased in mutant clones, opening the possibility that aberrant receptor signalling may participate in overgrowth of hpo-deficient tissue. Interestingly, however, although the polarity determinant Crumbs is required for the accumulation of apical proteins, this does not appear to significantly contribute to the overproliferation defect elicited by loss of Hippo signalling. Therefore, Hippo signalling controls growth and apical domain size by distinct mechanisms.

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Key words: Hippo signalling, Polarity, Proliferation

Introduction

In an epithelium, the optimisation of contact between neighbouring cells arises through the polarised architecture of each cell, which have several membrane domains separated by distinct types of cellular junctions (Tepass et al., 2001). In Drosophila epithelia, the membrane of a cell is divided in apical and basolateral domains. Those domains are separated from each other by the zonula adherens (ZA), an actin-rich region that forms a belt around the cell and where adherens junctions (AJs) are located (see supplementary material Fig. S1G). The main components of the ZA are the transmembrane adhesion protein Drosophila E-cadherin [DE-cad; also known as Shotgun (Shg)] and its scaffold protein Armadillo (Arm), the homolog of β-catenin.

The apical domain of the cell is subdivided into a free apical domain and the subapical region (SAR). The free apical domain is on the cell surface that is facing the external milieu. It is composed of a brush of microvilli whose formation depends on the apical localisation of the cadherin Cad99C (D’Alterio et al., 2005; Schlichting et al., 2006). Just below this free apical domain, the SAR is characterised by the presence of two apical polarity complexes (Tepass et al., 2001). The Bazooka (Baz) complex contains proteins such as aPKC (atypical protein kinase C), Par6 (Partitioning defect 6), Baz (the homolog of Par3), and the small GTPase Cdc42. The Crumbs (Crb) complex is composed of the transmembrane protein Crb, Stardust (Sdt) and Drosophila Patj.

Another type of cell-cell junction, the septate junction (SJ), is located basally to the ZA. The Scribble (Scrib) polarity complex, which consists of Scrib and Disc large (Dlg), is localised to the SJs, whereas the Lethal (2) giant larvae (Lgl) protein is present on all the lateral membranes (Tepass et al., 2001). These polarity determinants are highly conserved in mammals (Knust and Bossinger, 2002; Nelson, 2003).

How the polarity complexes localise at the appropriate plasma membrane compartment or how they regulate polarity remains unclear, but a hierarchy between them has started to emerge (Bilder et al., 2003; Tanentzapf and Tepass, 2003). The Baz complex appears to be the primary apical determinant. The Scrib complex prevents the assembly of the Baz complex in the basolateral part of the cell. In turn, the Baz complex antagonises the function of the Scrib complex by recruiting the Crb complex. These reciprocal inhibitions ensure a strict separation of the apical and basolateral domain. The Lgl protein has a unique role in the establishment and maintenance of this apicobasal polarity. On the one hand, localisation of Lgl to the basolateral membrane requires the Scrib complex (Bilder et al., 2000). On the other hand, Lgl is excluded from the apical membrane through its phosphorylation by aPKC (Betschinger et al., 2003; Plant et al., 2003). Finally, Lgl antagonises Crb function (Bilder et al., 2003; Tanentzapf and Tepass, 2003). Thus, the Lgl protein seems to act as an integrator of both signals from the apical and basolateral domains. Lgl, scrb and dgl are also known to function as tumour suppressor genes (Bilder et al., 2000), supporting the notion of a link between loss of polarity and overproliferation.

The Hippo pathway regulates tissue growth and proliferation by promoting cell cycle exit and apoptosis. The core of this pathway
is composed of two kinases, Hippo (Hpo) and Warts (Wts), and of the scaffold proteins Salvador (Sav) and Mob as Tumour Suppressor (Mats) (Harvey and Tapon, 2007). Activation of the pathway inhibits the activity of the Yorkie (Yki) transcriptional co-activator, triggering the downregulation of its target genes (Cyclin E, the Drosophila Inhibitor of Apoptosis 1 and the bantam miRNA) (Dong et al., 2007; Huang et al., 2005). The atypical cadherin Fat (Ft) as well as the FERM proteins Merlin (Mer) and Expanded (Ex) have been linked with the activation of this pathway, although the mechanistic details of this process remain unclear (Bennett and Harvey, 2006; Cho et al., 2006; Hamaratgolu et al., 2006; Silva et al., 2006; Willecke et al., 2006). Recently, the transcription factor Scalloped has been shown to bind to Yki and mediate DIAP1 regulation (Goulev et al., 2008; Wu et al., 2008; Zhang et al., 2008; Zhao et al., 2008a). Like wts, hpo and sav, mutations in ft, in Mer and ex, as well as yki gain-of-function, induce hyperplastic growth. The mutant cells have a proliferative advantage over wild-type cells but are able to differentiate. As a result, imaginal discs with mutant clones show outgrowths with extra folding of tissue but maintain their epithelial nature. In addition, adult epithelial cells lacking the activity of the Hpo pathway have hypertrophic apical domains (Justice et al., 1995; Wu et al., 2003).

The consequences of this apical hypertrophy have not been characterised, leading us to investigate in more detail the cell polarity defect elicited by Hpo pathway inactivation and its impact on cell proliferation. In the wing imaginal disc epithelium, we observed an increase of the apical polarity proteins aPKC, Crb and DE-cad which belong respectively to the Baz and Crb polarity complexes, and to the AJs, as well as an increase of the cell surface receptor Notch (N), when Hpo pathway activity is compromised. Basolateral markers (Dig and Dystroglycan) are not affected. The phenotype is characteristic of the Hpo pathway since apical hypertrophy is not observed when other pathways controlling cell growth or inducing cell sorting are compromised. Furthermore, this increase in apical polarity protein localisation is correlated with a hypertrophy of the apical domain and AJs. Interestingly, although accumulation of apical proteins is dependent on the apical determinant Crb, it is not required for overgrowth in response to loss of wts. Thus, the apical hypertrophy and overproliferative phenotypes of hpo-deficient cells are two distinct processes, suggesting that there is no straightforward link between an increase of surface receptors and an increase in signalling output.

Results

Generation of a hpo null allele

Different groups have generated a variety of hpo mutant alleles using chemical mutagenesis (Harvey et al., 2003; Jia et al., 2003; Udan et al., 2003; Wu et al., 2003). We had noted in previous studies that the various alleles differed considerably in strength (Polesello et al., 2006). We therefore decided to clarify the relative properties of the strongest known hpo alleles. First, we generated a null allele of hpo, hpo5.1, by imprecise excision of the P-element G3315 inserted in the 5′UTR of the hpo gene; supplementary material Fig. S1A). This excision allele, which removes most of the hpo coding sequence, represents a bona fide protein null for hpo. To test the strength of hpo5.1, we compared it with three other hpo alleles. The presumed null allele hpoBF33 is a point mutation introducing a premature stop codon in position 174, resulting in the production of a truncated protein (Jia et al., 2003). hpo42-47 is an in-frame deletion of six amino acids (166-171) in the Hpo kinase domain, N166 being required for ATP binding (Wu et al., 2003). hpoBF33 is a single amino acid substitution of a critical amino acid (G181E) in the kinase domain (Jia et al., 2003). Thus, the alleles hpo42-47 and hpoBF33 produce kinase-dead versions of Hpo.

Using an antibody directed against the C-terminal part of the Hpo kinase domain (Polesello et al., 2006), we examined Hpo staining in clones of cells mutant for each of the four hpo alleles described above. Mutant cells carrying the hpo5.1 mutation, or the hpoBF33 mutation, show no Hpo staining above background (supplementary material Fig. S1B,B′,D′). The antibody allows us to distinguish cells carrying one or two copies of wild-type (WT) hpo (one or two copies of GFP, respectively; supplementary material Fig. S1B,B′). By contrast, Hpo staining is not reduced in hpo5.1 or hpo42-47 clones (supplementary material Fig. S1C,C′,E,E′). The staining even appears more intense in some clones. This could be due either to increased Hpo stability in those mutants, or to altered localisation of the kinase-dead proteins. We also generated clones of hpo mutant cells in the eye (eyFLP driver), and assessed the viability of flies carrying those clones as a measure of the relative strength of the alleles. The expected fraction of flies containing mutant clones should be 50% of the total population. hpo42-47 or hpo5.1 clone-containing flies represented only 2.1% (4/190) or 13.1% (28/214) of the population, respectively, whereas hpo5.1 or hpoBF33 clone-containing flies were 34.3% (34/99) or 36.4% (52/143) of the population, respectively (supplementary material Fig. S1F). This result indicates that kinase-dead alleles (hpo5.1 and hpo42-47) have a stronger effect than null alleles (hpoBF33 and hpo42-47). This suggests that the kinase-dead alleles may have a dominant-negative effect, possibly interfering with activation of the downstream kinase Wts. Nevertheless, hpo5.1 and hpo42-47 behaved similarly in all subsequent experiments.

Disruption of the Hpo pathway elicits accumulation of apical and AJ proteins in imaginal disc cells

We first tested whether Hpo is required for normal distribution of the polarity complexes by generating FLP/FRT mitotic clones of mutant cells in wing imaginal discs. In hpo mutant cells, Cad99C, which localises to the apical microvilli (D’Alterio et al., 2005; Schlichting et al., 2006), shows a brighter and broader staining (Fig. 1A,A′). This indicates that the microvillus domain of the hpo mutant cells is larger than that of wild-type (WT) cells, since Cad99C overexpression is sufficient to induce growth of the apical microvilli (D’Alterio et al., 2005; Schlichting et al., 2006). As for Cad99C, stainings for aPKC and Crb revealed that, when hpo is mutated, there is an increased staining for members of the two apical polarity complexes (Fig. 1B-C′). The membrane domains where those proteins are localised also appear to be broader (see supplementary material Fig. S2A,A′,C,C′,G,G′ for XY sections and higher magnification). Phospho-Moesin (P-Moe), which co-immunoprecipitates with Crb in embryos (Medina et al., 2002), showed a similar pattern (Fig. 1D,D′); see supplementary material Fig. S2D,D′ for XY section), as well as the apically localised Notch receptor (supplementary material Fig. S3B,B′).

The AJs in hpo mutant cells are also characterised by increased protein localisation at the membrane, visualised by staining for Arm and DE-cad (Fig. 1E-F′; supplementary material Fig. S2E-F′ for XY sections). By contrast, the basolateral complexes are not affected by loss of Hpo signalling, as visualised by Dlg (lateral marker) and Dystroglycan (basal marker) stainings (Fig. 1G,H). This indicates that kinase-dead alleles (hpo5.1 and hpo42-47) have a stronger effect than null alleles (hpoBF33 and hpo42-47). The staining even appears more intense in some clones. This could be due either to increased Hpo stability in those mutants, or to altered localisation of the kinase-dead proteins. We also generated clones of hpo mutant cells in the eye (eyFLP driver), and assessed the viability of flies carrying those clones as a measure of the relative strength of the alleles. The expected fraction of flies containing mutant clones should be 50% of the total population. hpo42-47 or hpo5.1 clone-containing flies represented only 2.1% (4/190) or 13.1% (28/214) of the population, respectively, whereas hpo5.1 or hpoBF33 clone-containing flies were 34.3% (34/99) or 36.4% (52/143) of the population, respectively (supplementary material Fig. S1F). This result indicates that kinase-dead alleles (hpo5.1 and hpo42-47) have a stronger effect than null alleles (hpoBF33 and hpo42-47). This suggests that the kinase-dead alleles may have a dominant-negative effect, possibly interfering with activation of the downstream kinase Wts. Nevertheless, hpo5.1 and hpo42-47 behaved similarly in all subsequent experiments.
This suggests that the broader and more intense apical stainings observed in cells lacking hpo do not result from a mixing of the different apical polarity subdomains but might be caused by an enlargement of each subdomain of the apical region.

The accumulation of DE-cad and some apical signalling receptors had been noted for ft and mer;ex clones, respectively (Feng and Irvine, 2007; Jaiswal et al., 2006; Maitra et al., 2006). However, this was not believed to be a general property of Hpo pathway components. We therefore proceeded to test whether every level of the Hpo pathway showed the same disruption in the distribution of the polarity complexes. Clones of cells mutant for mer;ex or ft (Fig. 2A-A', data not shown), which have been suggested to be upstream elements of the network, as well as clones of cells mutant for wts (Fig. 2B-B'), the Hpo downstream kinase, were generated. As in hpo mutant clones, cells mutant for those genes show a brighter and broader staining for apical complex members, as well as a broadening of the Notch (N) receptor staining (supplementary material Fig. S3A-C'), but a normal distribution of basolateral markers. Overexpression clones for Yki, the downstream effector of the pathway whose gain of function mimics Hpo pathway loss of function, yielded the same phenotype (Fig. 2C-C'). Furthermore, activating the Hpo pathway induces the reverse phenotype as cells overexpressing ex or hpo show a reduction in apical localisation of aPKC (Fig. 2D,D',F,F'), DE-cad (Fig. 2E,E') and N (supplementary material Fig. S3F,F').

Hpo pathway mutant cells have apical membrane and AJ hypertrophy

The observed increase of apically localised polarity proteins in Hpo pathway-deficient cells could be the cause of the reported apical bulging observed by transmission electron microscopy (Justice et al., 1995) and scanning electron microscopy (Wu et al., 2003) in adult epidermal cells mutant for wts and hpo. We decided to investigate this further in wing imaginal discs by correlative transmission electron microscopy (TEM). To examine whether the apical domain of cells lacking a functioning Hpo pathway was expanded, we dissected third instar imaginal discs overexpressing yki and gfp under the control of the engrailed (en) promoter. The overgrown yki-overexpressing compartment can be identified using GFP (Fig. 3A). The discs retain their gross morphology after embedding in resin (Fig. 3B; compare with 3A). Thus, by correlating the transmitted light and GFP image with the TEM micrographs, the yki-overexpressing cells can be identified in transverse sections of the resin-embedded sample (Fig. 3C, transverse section cut across the region indicated by the dotted red line drawn on Fig. 3A,B) and it is possible to compare characteristics of control and yki-overexpressing cells in the same TEM section. The thickness of the wing pouch region appeared slightly reduced in the yki-expressing area, which might indicate a mild loss of stratification as a result of the apical polarity defect.

AJs are clearly identifiable in high magnification images of the apical region in control WT cells and yki-overexpressing cells (Fig. 3D,E), and are similar in morphology to embryonic AJs (Bachmann et al., 2008). To monitor apical hypertrophy, we measured AJs (see supplementary material Table S1). The mean size of AJs in WT cells is 411.70 nm, compared with 735.03 nm for those in yki-overexpressing cells (Fig. 3F). Thus, the AJs of yki-overexpressing cells are 1.78 times wider than those of WT cells.

The total surface of the apical domain is much more difficult to accurately determine than AJ length because of the presence of the microvilli. However, because of the cylindrical nature of
imaginal disc cells, we can consider that the apical surface is proportional to the product of the width by the length of the apical domain. We therefore measured cell width (W) at the AJs and the distance (L) between the AJ and the top-most apical membrane level (Fig. 3D,E). We then calculated the product PLW of those two values, which gives us a relative measure of subapical region size.

yki-overexpressing cells have a significantly larger apical domain [PLW(yki)=10.6×10^6] compared with control cells [PLW(control)=5.1×10^6] (Fig. 3G; for details, see supplementary material Table S1). Together with our immunofluorescence data, this suggests that deregulation of the Hpo pathway elicits an accumulation of apical and AJ proteins correlated with an increase in apical domain size and AJ width.

The accumulation of apical determinants resulting from loss of Hpo signalling may involve new Yki target genes

Yki is a transcriptional co-activator. It is therefore probable that the apical domain hypertrophy we observe in Hpo pathway loss-of-function cells is elicited by misregulation of one or more Yki target genes. Combined overexpression of the known Yki targets DIAP1, cycE and bantam (ban) increases the proliferation rate (Nolo et al., 2006) but overexpressing those three targets under the control of...
the patched promoter does not lead to an increase in aPKC staining at the apical domain of the cells (supplementary material Fig. S4B,B'). However, overexpressing Yki with the same promoter does induce the accumulation of apical proteins (supplementary material Fig. S4A,A'). This suggests that there could be another transcriptional target of Yki responsible for the apical hypertrophy.

Consequently, we determined whether any of the main polarity genes are targets of the Hpo pathway by conducting quantitative RT-PCR experiments on yki-overexpressing imaginal discs and on control discs (Fig. 4A). The yki-overexpressing discs had a robust increase in yki mRNA level compared with control discs. mRNA levels of ex, a known transcriptional target of the Hpo pathway (Hamaratoglu et al., 2006), were approximately threefold higher, as expected. We proceeded to measure mRNA levels for aPKC, crb, shg and arm. Whereas the crb mRNA level did not change in yki-overexpressing discs (1.04±0.14 fold), shg mRNA increased substantially [1.39±0.14 fold; P(yki-control)=0.013]. aPKC and arm mRNA levels were slightly, but not significantly, elevated (respectively 1.29±0.12 fold and 1.37±0.19 fold). Since the increase in ex mRNA, a well-documented target of the Hpo pathway, is relatively modest, it is possible that the effect on aPKC and arm mRNA levels might be biologically relevant but too subtle to be detected as significant in this experiment.

crb and shg transcription can also be visualised using a crb-lacZ and a shg-lacZ enhancer trap (Herranz et al., 2006; Tepass et al., 1996). crb is mainly expressed in a broad stripe at the dorsoventral boundary and in a fainter band at the anteroposterior boundary of the disc (Fig. 4B) (Herranz et al., 2006). In discs containing hpo clones (Fig. 4C-C”), a slight increase in staining could occasionally be observed (arrow) but the overall pattern of crb-lacZ was not significantly changed. By contrast, the shg enhancer trap, which has a more uniform expression in the disc, showed a marked increase in wts mutant cells (Fig. 4D,D’). Thus, for the two genes tested, the enhancer traps recapitulate the qRT-PCR results. ft clones were also shown to have elevated shg-lacZ (Jaiswal et al., 2006). Furthermore, in the first intron of the shg gene (position 369 to 395) we found a sequence with a high homology to the Hpo response element (HRE) (Wu et al., 2008) (Fig. 4E). The Scalloped-binding site in this putative shg HRE is conserved in other Drosophila species (data not shown). This suggests that shg may be directly regulated by the activity of Yki and Scalloped, prompting us to investigate, in more detail, the role of DE-cad in apical hypertrophy.

Our TEM analysis suggested that the AJs are wider than normal but are properly formed in yki-overexpressing cells (Fig. 3F). Furthermore, transverse sections of wing imaginal discs stained for DE-cad and Arm show a proper junctional co-localisation of both proteins (supplementary material Fig. S5A-A’). Finally, the level of DE-cad in Rab5/7/11-positive vesicles appears normal in hpo clones (supplementary material Fig. S5B,B’; and data not shown).

These data suggest that in Hpo-pathway-deficient cells excessive DE-cad is correctly targeted to the membrane. To study whether the increase in DE-cad in hpo mutant cells is sufficient to induce apical hypertrophy, we overexpressed shg under the control of the patched (ptc) promoter. This elicits a duplication of the notum (data not shown) through titration of Arm by excess DE-cad, resulting in a wingless loss-of-function phenotype (Sanson et al., 1996). To circumvent this problem, we expressed both shg and arm under the control of the patched promoter. Cells overexpressing those apical polarity determinants do not show an increase in aPKC staining (supplementary material Fig. S5C,C’). Occasionally, shg- and arm-overexpressing cells delaminate and lose polarity, as seen by membrane staining of aPKC around the whole cell surface (supplementary material Fig. S5D,D’). These results show that shg and arm overexpression does not phenocopy Hpo pathway loss of function and suggest that increases of DE-cad and Arm in Hpo-pathway-deficient cells is not sufficient to induce apical hypertrophy. Thus, whereas shg might be a direct target of the Hpo pathway, the apical expansion phenotype may involve additional Yki targets.

The increase in apically localised proteins is not a general consequence of cell sorting defects or overgrowth

Several growth-promoting manipulations in Drosophila epithelial cells have been shown to induce ‘cell-sorting’ defects. In these situations, the mutant cells appear to adhere preferentially to neighbours of the same genotype and tend to minimise contacts with WT neighbours, leading to formation of round clones. This property, which is shared by Hpo pathway mutants (Harvey and Tapon, 2007), cells expressing oncogenic Ras (Prober and Edgar, 2000) and an activated form of the Dpp receptor Thickveins (Tkv) (Nellen et al., 1996), remains little understood but is believed to result from altered cell-cell adhesion properties. We therefore wished to determine whether the apical protein accumulation in Hpo
pathway mutants is a consequence of this cell-sorting defect. We generated \(ras^{12}\)-overexpressing cells in the wing disc (supplementary material Fig. S4C,C’). aPKC staining in those cells was not detectably changed compared with WT cells. We conclude that increased levels of apical determinants is a specific consequence of loss of Hpo signalling, and is not common to all cells with a sorting defect.

We tested whether accumulation of apical proteins is merely a consequence of the overgrowth elicited by loss of Hpo signalling or a specific consequence of loss of this pathway. Overexpressing \(cyclin\ D\) \((cycD)\) and \(cyclin-dependent\ kinase\ 4\) \((cdk4)\) in the wing imaginal disc leads to an increase in cell proliferation by accelerating cell growth (Datar et al., 2000). However, those cells do not show a brighter or broader staining of aPKC (supplementary material Fig. S4D,D’). Furthermore, as previously discussed, overexpressing the known Hpo pathway targets \(cycE\), \(DIAP1\) and \(ban\) strongly promotes proliferation, but does not lead to an increase in aPKC staining at the apical domain of the cells (supplementary material Fig. S4B,B’). Thus, the apical hypertrophy seen in cells mutant for the Hpo pathway is not a general property of all overproliferating tissues.

Signalling consequences of apical determinant accumulation

Maitra et al. reported that several receptors, such as Smo, Egfr and N accumulate on the surface of \(mer;ex\) double clones, probably because of an imbalance between endo- and exocytosis in these cells (Maitra et al., 2006). The same study also contends that N signalling is disrupted in \(hpo\) clones in the ovarian follicular epithelium (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). We therefore decided to further explore N signalling in wing discs.

We confirmed that N is apically upregulated in all Hpo pathway members that we tested, and that this phenotype is not specific to \(mer;ex\) clones (supplementary material Fig. S3). We looked at the expression ofCut, a direct target of N that is expressed in a thin stripe at the dorsoventral (DV) boundary of the wing disc (de Celis and Bray, 1997; de Celis et al., 1996). Cut staining can be used to monitor an increase in N activity (Childress et al., 2006). We therefore generated clones for \(mer;ex\) or \(hpo\). When the Cut expression domain crossed mutant cells, we did not observe a broader Cut stripe (Fig. 5A-B’). On the contrary, there appeared to be a slight reduction in its width. To confirm that result, we also used the \(Gre(H)-lacZ\) reporter to monitor N target gene expression (Furriols and Bray, 2001). In WT wing discs, this reporter is highly expressed in a stripe at the DV boundary, which is the region of highest N activity. It is also expressed more faintly in patches in the pouch of the wing disc, a region of lower N activity (Fig. 5C). Clones of \(wts\) mutant cells expressing the \(Gre(H)-lacZ\) reporter and located in the pouch clearly showed a decrease in \(β\)-galactosidase (\(β\)-gal) staining. In rare clones that straddle the DV boundary, there was a decrease in the high N activity region (Fig. 5D-D’). The \(E(sp)\_lacZ\) reporter gives similar results to \(Gre(H)-lacZ\) (not shown). This suggests that, as in ovaries, N signalling is impaired in Hpo pathway loss-of-function wing disc clones. Thus, whereas apical proteins, including signalling receptors, accumulate on the surface of mutant cells, depending on the receptor, this may result in increased activation (if the receptor signals at the surface) or decreased activation (if the receptor needs to be internalised to signal).

Is apical determinant accumulation required for cell proliferation?

The fact that receptors aberrantly accumulate on the surface of Hpo-pathway-deficient cells opens the possibility that the overproliferation in Hpo pathway mutants is due, at least in part, to apical hypertrophy leading to abnormal signalling (Maitra et al., 2006). For example, excess Egfr activation would be predicted to promote both proliferation and cell survival (Baker and Yu, 2001; Dominguez et al., 1998). Finally, Crb overexpression can induce hyperplastic growth in wing discs (Lu and Bilder, 2005). To determine the relationship between apical expansion and overproliferation, we took advantage of the fact that \(crb\) mutant cells in the wing disc do not lose apicobasal polarity and delaminate. Furthermore, TePas and colleagues reported that Crb is required for apical membrane growth in the retina (Pellicka et al., 2002). We therefore reasoned that removing Crb might prevent Hpo pathway-induced apical membrane hypertrophy.

As anticipated, apical proteins failed to accumulate in \(crb/wts\) cells. NICD and aPKC stainings are not fully homogeneous in \(crb/wts\) clones, with occasional clones still showing slightly increased staining in a row of cells at the periphery (supplementary material Fig. S3D,D’), whereas in most of the clones, stainings are weaker than in WT cells, as can be seen in transverse sections (Fig. 6B; supplementary material Fig. S3D,D’). As expected, aPKC and NICD apical stainings are slightly reduced in \(crb\) clones (Fig. 6C; supplementary material Fig. S3E,E’).

To quantify the extent of the apical determinant increase in \(wts\), \(hpo\) and \(crb/wts\) mutant cells, we generated \(hpo\), \(wts\) and \(crb/wts\) clones in wing discs and measured the staining intensity ratio between mutant cells (no GFP) and non-mutant cells (one or two copies of GFP). We calculated this ratio (\(r\)) for aPKC, NICD and Dlg stainings (Fig. 6D; supplementary material Table S2). As expected, for discs containing control (WT) clones, the ratio approached 1 for the three stainings. Dlg staining intensity in all conditions tested was not significantly altered, suggesting that the septate junctions are not affected. aPKC and N levels were increased in \(hpo\) and \(wts\) mutant cells compared with WT cells confirming our previous observations in transverse sections (Fig. 1, Fig. 2B-B’). NICD and aPKC stainings are not fully homogeneous in \(crb\) clones (Fig. 6C; supplementary material Fig. S3E,E’). In \(crb/wts\) clones, the levels of both aPKC and NICD were at WT levels, indicating that loss of

\[\text{Fig. 5. Downregulation of Notch activity in Hpo signalling-deficient cells.}\]
\[\text{(A-D') XY sections of wing imaginal discs. (A', B') Merged images of A and B with GFP (green). (D') The merged images of D (red) and D' (green). Scale bar: 20\,\mu\text{m}}\]

\[\text{Supplementary material Fig. S4C,C'.} \]

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\[\text{To quantify the extent of the apical determinant increase in wts, hpo and crb/wts mutant cells, we generated hpo, wts and crb/wts clones in wing discs and measured the staining intensity ratio between mutant cells (no GFP) and non-mutant cells (one or two copies of GFP). We calculated this ratio (r) for aPKC, NICD and Dlg stainings (Fig. 6D; supplementary material Table S2). As expected, for discs containing control (WT) clones, the ratio approached 1 for the three stainings. Dlg staining intensity in all conditions tested was not significantly altered, suggesting that the septate junctions are not affected. aPKC and N levels were increased in hpo and wts mutant cells compared with WT cells confirming our previous observations in transverse sections (Fig. 1, Fig. 2B-B', Fig. 6A; supplementary material Fig. S3B-C'). Both stainings were slightly less in crb mutant cells (Fig. 6C; supplementary material Fig. S3E,E'). In crb/wts clones, the levels of both aPKC and NICD were at WT levels, indicating that loss of}\]
crb can indeed rescue the apical determinant and receptor accumulation observed in wts mutant tissue.

To determine if apical protein accumulation has an impact on the overgrowth phenotype of Hpo pathway loss of function, we compared the growth rates of clones mutant for crb, wts or crb/wts, in density-controlled crosses. When mutant clones and WT twin spots are generated using the FLP/FRT system and a heat-shock Flipase transgene, both mutant (GFP-negative) and WT (two copies of GFP) cells are born at the same time from the same recombination event. Thus, comparing the size of the mutant and WT areas after a fixed time interval (72 hours) allows us to compare the growth rate of mutant cells with that of normal cells. We therefore dissected wing discs containing clones of the relevant genotypes, acquired images in the GFP channel and calculated the ratio \( r_{CT} \) between the total clonal area and the total twin spot area for the different genotypes (Fig. 6E,F; supplementary material Table S3).

As expected, the ratio approached 1 for discs containing control (WT) clones \( r_{CT(\text{control})} = 1.08 \). Discs containing crb clones have a ratio very similar to that of control clones, showing that crb mutant cells have neither a growth advantage nor a growth defect in the wing. As previously shown, wts clones have a growth advantage over WT tissue \( r_{CT(wts)} = 2.93; \ P(wts-\text{control}) = 1.29E-08 \). Finally, clones of crb/wts mutant cells also have a growth advantage over their twin spot \( r_{CT(crb/wts)} = 2.96; \ P(crb/wts-\text{control}) = 6.65E-09 \). Importantly, the growth advantages of wts and crb/wts clones are almost identical \( P(wts-crb/wts) = 0.95 \). In addition, the rate of apoptosis is the same in wts clones and crb/wts double clones, as assayed by anti-active caspase-3 staining (not shown). These results suggest that the apical hypertrophy is not necessary for the overgrowth phenotype elicited by disruption of Hpo signalling.

Apical constriction occurs in hpo or wts mutant cells but cannot account for apical determinant accumulation

The hypertrophy of the apical domain we observe in the absence of wts or hpo could either be due to a net expansion of the apical membrane or a change in cell shape. For instance, constriction at the ZA could increase the apparent levels of aPKC staining by inducing the bulging out of the apical membrane. Our TEM measurements (Fig. 3H; supplementary material Table S1) took this possibility into account since we measured the width as well as the length of the apical domain. Nevertheless, to confirm this in an independent manner, we quantified apical constriction in wts, hpo and crb/wts mutant clones. We generated positively labelled clones of cells mutant for hpo, wts or crb, wts stained for DE-cad and filamentous actin (supplementary material Fig. S6A-C). We calculated the ratio \( r_{ACS} \) between apical cell surface at the level of the ZA in mutant areas and in WT areas (supplementary material Fig. S6D, Table S4). hpo and wts mutant cells were mildly constricted compared with WT cells since the apical cell surface ratio was below one \( r_{ACS(hpo)} = 0.84; \ P(hpo-\text{wts}) = 0.88 \). Thus, apical constriction could participate in the apical hypertrophy phenotype. However, in crb/wts clones, which do not show apical protein accumulation, we observed a similar constriction to that in wts cells \( r_{ACS(crb/wts)} = 0.92; \ P(crb/wts-\text{wts}) = 0.45 \). We noted that some areas in crb/wts clones had discontinuous DE-cad staining, preventing us from estimating cell number in these areas, even though cell densities appeared even higher than in other parts of the clones. Thus, the apical constriction in crb/wts clones is probably slightly underestimated, suggesting that, despite the fact that aPKC staining is normal in crb/wts clones, the cells are in fact more constricted than in wts tissue. As suggested by the TEM measurements, these results show that, whereas ZA constriction may contribute to the apical membrane phenotype, this is primarily due to an increase in apical determinants.

**Discussion**

**Polarity and the Hpo pathway**

We have examined, at the molecular level, apicobasal polarity in cells mutant for the Hpo tumour-suppressor pathway. We show that cells in which the Hpo pathway is inactivated stain more intensely...
for apical polarity, ZA components and the N receptor, which is correlated with an increase in apical domain size and adherens junction width. By contrast, basal polarity proteins are unaffected (Figs 1, 2 and 3). This altered polarity is manifest in disruptions at every level of the Hpo pathway, from upstream elements (e.g. mer;ex clones) to cells overexpressing Yki. Though wts and hpo mutant cells are mildly constricted, our results suggest that the apical hypertrophy phenotype is a consequence of increased levels of apical determinants (Fig. 3G; supplementary material Fig. S6). Increase in the apical domain staining of several cell surface receptors had been reported for mer;ex mutant clones (Maitra et al., 2006), leading to the suggestion that Ex and Mer control Hpo activity indirectly via other signalling pathways. Our data indicate that apical determinant accumulation is a general consequence of loss of Hpo signalling. This suggests that Ex and Mer are indeed bona fide members of the Hpo pathway.

How does apical hypertrophy arise?

We can suggest several possible causes for the apical protein expansion phenotype. First, loss of basal determinants such as Scrib or Dlg is known to increase the apical domain (Bilder and Perrimon, 2000). However, the basal polarity machinery appears undisturbed in hpo pathway clones, which rules out this possibility (Figs 1 and 2). Second, one or several polarity determinants may be transcriptional targets of Yki. At the very least, the fact that Yki overexpression (but not that of known pathway targets cye,E, DIAPI and bun) is sufficient for apical hypertrophy suggests that the effect of Hpo pathway loss of function on polarity is transcriptional (supplementary material Fig. S4). The shg gene, which encodes the ZA-associated protein DE-cad, could be a target, since shg transcriptional activity is increased in wts and ft clones as well as in yki-overexpressing discs (Fig. 4) (Jaiswal et al., 2006). In fact, a putative Hpo response element [HRE (Wu et al., 2008)] containing a conserved Scalloped (Sd) binding site is present in the shg first intron. Yki, with its partner Sd, could therefore be directly responsible for the increased transcriptional activity of shg. However, whereas increased DE-cad could account for broader junctions, it would not necessarily be predicted to lead to an expanded apical domain. Indeed, combined DE-cad and Arm overexpression is not sufficient to drive apical expansion (supplementary material Fig. S5). The apical determinant Crb has been reported to promote apical membrane growth by antagonising the FERM domain protein Yurt, although the mechanism remains to be defined (Laprise et al., 2006). Interestingly, our results indicate that Crb activity is necessary for apical protein accumulation upon loss of wts (Fig. 6). The mild increase in crb transcription we observe in some hpo clones may not be sufficient to explain this phenotype (Fig. 4). It will therefore be interesting to see whether Hpo signalling can modulate Crb or Yurt activity.

Finally, apical hypertrophy might occur as a result of an imbalance between exo- and endocytosis. This interesting possibility is supported by two lines of evidence. Firstly, mer;ex clones have decreased rates of N endocytosis (Maitra et al., 2006). Secondly, hpo mutant clones in ovarian follicle cells have ectopic accumulation of the endosomal sorting protein Hrs (HGF-regulated tyrosine kinase substrate) (Yu et al., 2008). Altered vesicle trafficking would be predicted to lead to inappropriate delivery of polarity proteins, which might account for apical determinant accumulation. So far, no known Yki targets could account for such a phenotype, but future research should resolve this important issue. It is interesting to note that many regulators of vesicle trafficking also appear to function as tumour suppressor genes in Drosophila (Harrisharan and Bilder, 2006).

Apical protein accumulation and cell surface signalling

mer;ex cells have been reported to increase apical levels of different signalling receptors such as Egfr, the Hedgehog receptor Smoothened and N (Maitra et al., 2006). We found that hpo or wts mutant cells had more N receptor at their apical membrane (supplementary material Fig. S3). This led us and others (Maitra et al., 2006) to speculate that the presence of more signalling receptors at the apical surface, where many signalling events occur, could participate in the hpo overgrowth phenotype by triggering ectopic signalling. However, the fact that crb/wts mutant cells overproliferate to the same extent as wts mutant cells while not having increased apical N or AKP4 suggests that apical hypertrophy and proliferation are separate processes (Fig. 6).

Does the presence of higher levels of receptor at the cell surface necessarily equate to increased signalling? Despite this accumulation of N receptors, N signalling is downregulated in follicle cells mutant for the Hpo pathway (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). Our results show that in epithelial imaginal wing cells, N signalling activity is decreased in Hpo pathway loss-of-function cells, despite increased N at the apical surface (Fig. 5; supplementary material Fig. S3). We can hypothesise that reduced N endocytosis blocks its cleavage by γ-secretase, which normally occurs in late endosomes and is a crucial step for N receptor activation (Le Borgne, 2006). Thus, depending on whether a receptor requires internalisation for activation or signalling, we can expect distinct outcomes (activation or blockage) in Hpo pathway mutant tissue.

Epithelial architecture and cancer

In human cancer, loss of polarity is a feature of invasive tumours (Wodarz and Nathke, 2007). Indeed, immortal cells mutant for scrib or dlg are highly invasive, suggesting that loss of polarity can promote invasion of surrounding tissue. By contrast, wts mutations failed to cooperate with oncogenic ras or scrib mutations in an eye disc invasion model (Igaki et al., 2006; Pagliarini and Xu, 2003). However, in ovarian follicle cells, wts mutations have been reported to induce invasion and to potentiate the invasive phenotype of dlg mutant cells (Zhao et al., 2008b). Thus, perturbation of epithelial architecture induced by loss of Hpo pathway signalling may well participate in the invasive behaviour of some tumour types.

Materials and Methods

Fly stocks

w; FRT42D,hpo1641/Cyo.P[act::GFP] and yw; FRT42D,hpo1641/Cyo.P[act::GFP] were gifts from Jin Jiang (University of Texas Southwestern Medical Center, Dallas, TX) (Jia et al., 2003); w; FRT42D,hpo1641/Cyo.P[act::GFP] and w; UAS-yki/TM3,SB were gifts from Duojian Pan (Johns Hopkins University School of Medicine, Baltimore, MD) (Huang et al., 2005; Wu et al., 2003); yw; FRT82B,wts1118/TM3,SB was a gift from Tian Xu (Yale University School of Medicine, New Haven, CT) (Xu et al., 1995); yw; Mer7,FR14A; P[UbxMer].P[UbxGFP].FRT40A/Cyo.P[act::GFP] and w; FRT40A/Cyo.P[act::GFP].fisdPMKRS/TM6B were gifts from Richard Fehon (University of Chicago, Chicago, IL) (Maitra et al., 2006); w; UAS: ex was obtained from Georg Halder (M. D. Anderson Cancer Center, Houston, TX) and is a construct by Peter Bryant (University of California, Irvine, CA) (Boedigheimer et al., 1997). yw;hsFLP: UAS: cye,D; UAS: cad4/SM6-68 was a gift from Bruce Edgar (Fred Hutchinson Cancer Research Center, Seattle, WA) (Datar et al., 2000); w; UAS: Cad.UAS: Arm.Cyo was a gift from Jean-Paul Vincent (National Institute for Medical Research, London, UK); w; E(gam)luc-iauc and w; Gre(H)lac-Z were gifts from Sarah Bray (University of Cambridge, Cambridge, UK); yw; P[act::GFP] and YCT1540 wts1118/Cyo.O (shg-lacZ in the text) was obtained from Emily Richardson (University College London, London, UK). w;hs(Rl)d; psc-gal4.UAS: GFP/Cyo was a gift from Barry Thompson (CRUK London Research Institute, London, UK). w; crb1660; M3 (crb-lacZ in the text) and w; FRTcrb1660/TM3.Sb were gifts from Marco Milan (Institut de Recerca Biomedica, Barcelona, Spain) (Herranz et al., 2001).
were acquired on a Zeiss LSM510 confocal laser scanning microscope (25×). After washes, tissues were mounted in Vectashield (Vector). Fluorescence images from Donald F. Ready (Purdue University, West Lafayette, IN) was used at 1/500, [a gift from Wu-Min Deng (Florida State University, Tallahassee, FL)] was used at 1/500, the galactosidase antibody (Promega) was used at 1/500 and NICD (C17.9C6) antibody, Fig. 3G). Cell width was measured at the AJs. Apical domain length was calculated from supplementary material Fig. S1), or

**Immunostainings**

Mosaic tissues were obtained with the FLP/FRT system with hsFLP drivers (see supplementary material Fig. S1), or hsFLP drivers in all other experiments. For loss-of-function experiments, 48-hour larvae were heat shocked for 50 minutes at 37°C and dissected 3 days after heat shock. For gain-of-function experiments (flip out), 72-hour larvae were heat shocked for 5-20 minutes at 37°C and dissected 2 days later. Some gain-of-function (supplementary material Fig. S4) experiments were carried out using ptc-gal4 drivers.

Tissues were processed as described by Polesello et al. (Polesello et al., 2006). β-galactosidase antibody (Promega) was used at 1/500 and NICD (C17.9C6) antibody, from Donald F. Ready (Purdue University, West Lafayette, IN) was used at 1/500. Anti-SH124 [a gift from W-M Deng (Florida State University, Tallahassee, FL] was used at 1/300. Anti-Arm (N27A1 from DSHB) antibody was used at 1/10, anti-βP (Santa Cruz) at 1/500 and anti-E-cad at 1/20. Anti-Cad99C [a gift from Dorothea Godt (University of Toronto, Toronto, Canada)] was used at 1/3000, anti-P-Moe [a gift from Dorothea Godt (University of Toronto, Toronto, Canada)] was used at 1/1000. Anti-Dig (4F3 from DSHB) was used at 1/500. Anti-Cut (2B10 DSHB) was used at 1/20. Anti-Rab5 was used at 1/1000 [a gift from Akira Nakamura (Riken Center for Developmental Biology, Kobe, Japan)]. Rhodamine-phallolidin (Sigma) was used at 1/500. Secondary antibodies (Rhodamine red X-conjugated donkey anti-rabbit, anti-rat or anti-mouse, Cy5-conjugated donkey anti-mouse; from Jackson ImmunoResearch) were used at 1/500. Hoechst 33342 was added for 20 minutes. After washes, tissues were mounted in Vectashield (Vector). Fluorescence images were acquired on a Zeiss LSM510 confocal laser scanning microscope (25× and 40× lenses, zoom 1 or 2), either as XY acquisitions or transverse sections (NX).

**Correlative transmission electron microscopy (TEM) of wing imaginal discs**

Third instar imaginal discs were dissected in fixing solution A (PBS + 4% PFA) and transferred on to an etched grid coverslip (Belco, 1916-91012) coated with poly-L-lysine to aid correlation between light and EM imaging. Transmitted light and GFP Z-stack images of the apical part of third instar imaginal wing discs containing WT, hpo, wts, or crb/wts clones and stained for apfk, NICD or Dlg, were taken with a p0.9 μm every 0.4 μm. Using the Bitplane Imaris imaging software, the intensity in all the Z-stack was assessed in different areas of mutant tissue (absence of GFP) or of non-mutant tissue (presence of GFP). For each disc, the staining intensity was measured in five to 15 areas of mutant tissue and in an equal number of non-mutant tissue areas. The chosen areas were representative of the heterogeneity of the tissue sample. As well as the intensity ratio between mutant and non-mutant tissue for each disc. Five discs were processed in the same way for each genotype and each staining. The statistical significance of the difference between WT clones and mutant clones was assessed by a Mann-Whitney non-parametric test. P < 0.01 when the difference was significant.

**Staining intensity ratio measurements**

For size measurement experiments, 50 L1 larvae (0–4 hour collections) per vial were collected from agar plates 24 hours after egg deposition and were grown until larval stage L3 before dissection. Heat shocks were as described above. Images of the discs were acquired with a 10× lens on a Zeiss LSM510 confocal laser scanning microscope and a pinhole of 5 μm. Measurements of the GFP negative area (clonal area) and two randomly chosen 2-3 μm2 areas were made by hand using Adobe Photoshop CS. Each area of interest was measured, with pixel as the unit, using the Polygonal Lasso Tool. All clonal areas of a disc were then added, and compared to the sum of the twin spot areas of the same disc. This process was repeated for several discs (5 ±0.16) and the results were averaged. The statistical significance of the difference between WT and mutant tissue was assessed by a Mann-Whitney non-parametric test. P < 0.01 when the difference was significant. Supplementary Table S2 presents the ratios, called r, of staining intensities for apfk, NICD and Dlg between clones of WT (Blank), hpo, wts or crb/wts cells, and non-mutant cells of the same wing disc.

**Standard conditions and size measurements**

For size measurement experiments, 50 L1 larvae (0–4 hour collections) per vial were collected from agar plates 24 hours after egg deposition and were grown until larval stage L3 before dissection. Heat shocks were as described above. Images of the discs were acquired with a 10× lens on a Zeiss LSM510 confocal laser scanning microscope and a pinhole of 5 μm. Measurements of the GFP negative area (clonal area) and two randomly chosen 2-3 μm2 areas were made by hand using Adobe Photoshop CS. Each area of interest was measured, with pixel as the unit, using the Polygonal Lasso Tool. All clonal areas of a disc were then added, and compared to the sum of the twin spot areas of the same disc. This process was repeated for several discs (5 ±0.16) and the results were averaged. The statistical significance of the difference between WT and mutant tissue was assessed by a Mann-Whitney non-parametric test. P < 0.01 when the difference was significant. Supplementary Table S2 presents the ratios, called r, of staining intensities for apfk, NICD and Dlg between clones of WT (Blank), hpo, wts or crb/wts cells, and non-mutant cells of the same wing disc.

**Quantification of apicidal concentration**

hpo, wts, or crb/wts clones positively labelled with GFP were generated and stained for F-actin and Arm to visualise the apical side of the cells. For five different clones of each genotype, the clonal area (GFP) was measured as well as the number of cells constituting it, thus allowing us to calculate the mean apical cell surface in that clone. The same procedure was followed for an area of non-mutant tissue close to the clone. We then calculated the ratio between the apical cell surface in the clonal area and the apical cell surface in an adjacent non-mutant area, which provides us with an index of apicidal cell concentration. The results were then averaged and the statistical significance of the difference between different mutant tissues was assessed by a Mann-Whitney non-parametric test. P < 0.01 when the difference was significant. Supplementary Table S4 presents the ratios, called r, of staining intensities for apfk, NICD and Dlg between clones of WT (Blank), hpo, wts or crb/wts cells.

**Genotypes**

Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51. Fig. 2B-B′, D′-D″, G′-H′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo/+.

Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51.

Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51.

Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51.

**Genotypes**

Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51. Fig. 2B-B′, D′-D″, G′-H′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51. Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51. Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51. Fig. 2A-A′, C′-C″, E′-F′, L′-I′: yw,hbsFLP/w; FRT42D, GFP/FRT42D, hpo. 51.
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