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The tumour-suppressor genes *NF2/Merlin* and *Expanded* act through *Hippo* signalling to regulate cell proliferation and apoptosis

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Merlin, the protein product of the Neurofibromatosis type-2 gene, acts as a tumour suppressor in mice and humans. Merlin is an adaptor protein with a FERM domain and it is thought to transduce a growth-regulatory signal. However, the pathway through which Merlin acts as a tumour suppressor is poorly understood. Merlin, and its function as a negative regulator of growth, is conserved in *Drosophila*, where it functions with Expanded, a related FERM domain protein. Here, we show that *Drosophila* Merlin and Expanded are components of the Hippo signalling pathway, an emerging tumour-suppressor pathway. We find that Merlin and Expanded, similar to other components of the Hippo pathway, are required for proliferation arrest and apoptosis in developing imaginal discs. Our genetic and biochemical data place Merlin and Expanded upstream of Hippo and identify a pathway through which they act as tumour-suppressor genes.

During normal development, the number of cells in growing tissues is controlled by regulating the generation of new cells through cell proliferation and by apoptosis, which eliminates excess or damaged cells that may be harmful to the organism[1,2]. Deregulation of these processes can lead to tumour formation[3]. Recently, a novel tumour-suppressor pathway that coordinately regulates cell proliferation and apoptosis was identified via genetic studies in *Drosophila*[4,5]. It is composed of the serine/threonine kinases Hippo (Hpo) and Warts (Wts); the adaptor molecule Salvador (Sav); Mats (Mob as tumour suppressor), a regulator of Wts; and Yorkie (Yki), a transcriptional coactivator[6–16]. Mutations in Hpo, Sav, Wts, Mats or overexpression of Yki result in overgrown tissues containing excess cells. Extra cells in these mutants are generated by deregulated cell proliferation and resistance to apoptotic stimuli that normally eliminate excess cells. All five proteins are highly conserved between flies and vertebrates and, in both systems, Hpo acts upstream of Wts[6–17]. However, factors that act upstream of Hpo remain to be identified.

Here, we identify two upstream components of the Hpo signalling pathway: the *Neurofibromatosis type-2 (NF2)* encoded protein Merlin (Mer), and a related protein Expanded (Ex). Mutations in the *NF2* tumour-suppressor gene underlie neurofibromatosis type-2, a familial cancer syndrome that shows development of tumours in the central nervous system[18–20]. Several lines of evidence further support the idea that Mer acts as a tumour-suppressor gene. For example, heterozygous *Nf2*

mutant mice spontaneously develop a wide range of highly metastatic tumours[21]; cultured *Nf2*-deficient cells fail to undergo contact-dependent growth arrest[22], and Mer overexpression suppresses cell proliferation[23,24]. Mer is a member of the protein 4.1 superfamily of adaptor proteins[18,19] and is thought to transduce a signal from membrane receptors to intracellular downstream components[20,24,25]. However, although Mer has been shown to interact with several proteins, including PAK1, CD44, HRS, PIKE-L, NHE-RF and ERM, how Mer functions as a tumour-suppressor gene is poorly understood[20,24–26]. We further investigated the function of Mer as a growth suppressor *in vivo*, taking advantage of the genetic tools that are available in *Drosophila*.

The growth-suppressing function of Mer is conserved in *Drosophila*, where it acts partially redundant with another FERM domain protein, Ex[27–29]. Mer and Ex colocalize to adherens junctions[22,27,30], where they may transduce a growth-suppressing signal from an unknown receptor[20,24,25]. Null mutations in *mer* and *ex* are lethal in *Drosophila*[29,31], but animals that are homozygous for hypomorphic mutations of either *mer* or *ex* survive and show slight overgrowth of adult structures, such as wings[29–31]. Mutations in *mer* and *ex* show dominant genetic interactions and clones that are doubly mutant for *mer* and *ex* have defects in growth control and differentiation[27]. However, the cellular basis for these phenotypes and the pathway through which *mer* and *ex* act as tumour-suppressor genes are not known[22,25,27].

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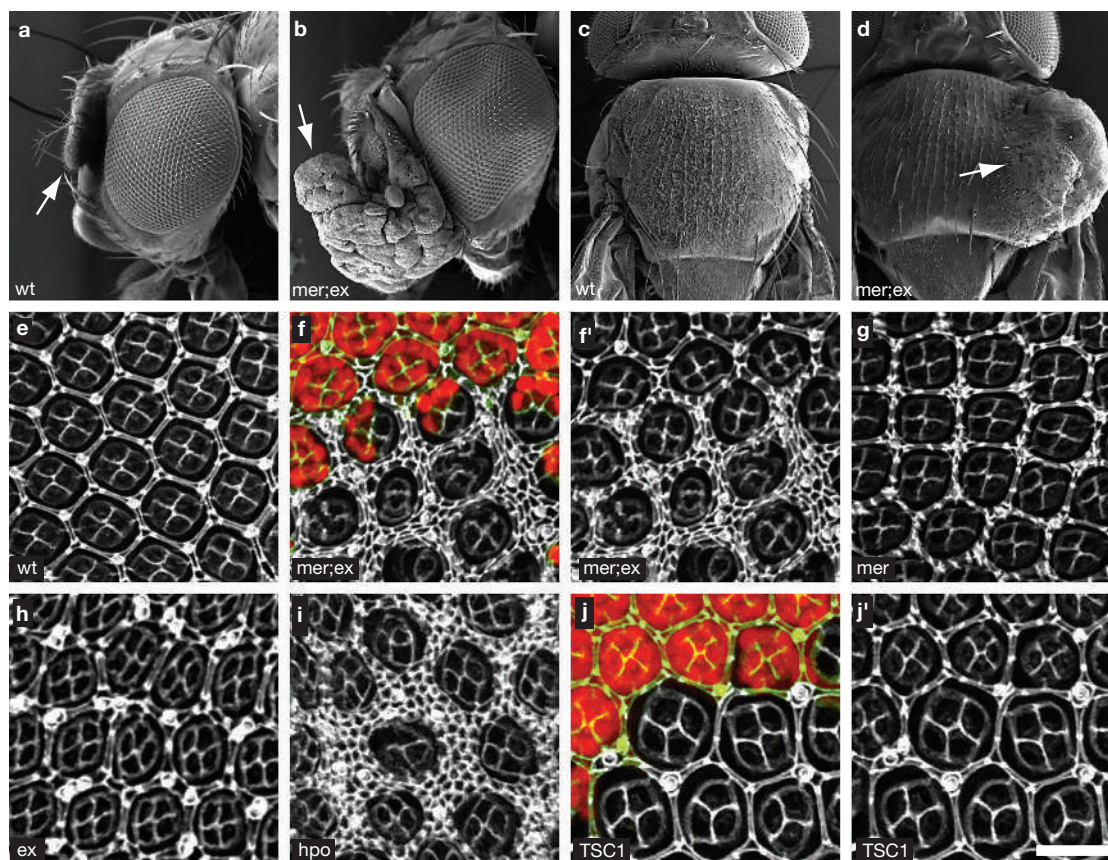
Figure 1 *merlin* and *expanded* regulate tissue size. (**a**, **b**) Scanning electron micrographs (SEMs) of a wild-type (wt) fly head (**a**) and a head with *mer⁴;exᵉ¹* double-mutant clones (**b**) in the antenna (arrow), resulting in massive tissue overgrowth. (**c**, **d**) SEM images of a wild-type thorax and a thorax with *mer⁴;exᵉ¹* double-mutant clones (arrow). (**e-j'**) Mid-pupal retinae stained with anti-Discs large (Dlg) antibodies that localize to apicolateral junctions and visualize cell outlines. (**e**) Wild-type retina. (**f, f'**) Retina with a *mer⁴;exᵉ¹* double-mutant clone marked by the absence of green fluorescent protein (GFP) expression (red in **f**). (**f'**) shows the Dlg staining only. The mutant area shows excess interommatidial cells. (**g**) *mer⁴* and (**h**) *exᴮᵠ* mutants display few extra interommatial cells. (**i**) *hpo⁴²⁻⁴⁷* and (**j, j'**) *TSC1ᴵᵠ⁶⁹* mutant retinae. Absence of GFP (red) marks the mutant clone in (**j**) and (**j'**) shows Dlg only. The phenotype of *mer⁴;exᵉ¹* double-mutant tissues is similar to *hpo⁴²⁻⁴⁷* but distinct from *TSC1ᴵᵠ⁶⁹* mutants. Scale bar (**j'**), 20 μm.

RESULTS

Mutations in *merlin* and *expanded* cause tissue overgrowth

To define the functions of *mer* and *ex* in growth control and to identify the pathway downstream of Mer and Ex, we analysed the phenotypes of *mer;ex* double-mutant clones during development (see Methods). For this analysis, we used previously identified null alleles of *mer* and *ex*[28,29], as well as new *ex* alleles that we isolated in a genetic screen (see Supplementary Information, Fig. S1). We found that clones of cells doubly mutant for *mer* and *ex* produced pronounced outgrowths in diverse adult structures, such as antennae, thorax, wings and legs (Fig. 1a–d; and data not shown). Therefore, *mer* and *ex* are general growth regulators that are required to restrict the size of adult structures.

To determine the cause of the overgrowth phenotypes, we studied cell proliferation and apoptosis in tissues lacking Mer and Ex function. We focused our analysis on the developing eye, where defects in the regulation of cell proliferation and apoptosis are readily detectable[32]. The *Drosophila* eye develops from the eye imaginal disc, a single-cell-layered epithelial sheet that grows during the larval stages[33]. After photoreceptor, cone and pigment cells are determined, extra cells are eliminated by apoptosis[32], generating a perfect lattice of the different cell types, which can be visualized during the pupal stage (Fig. 1e). In *mer;ex* double-mutant cell clones, this pattern is disrupted and the retina contains a large excess of interommatidial pigment cells (Fig. 1f, f'). The size of mutant cells appeared normal, although the morphology of photoreceptors and the numbers of cone cells were often abnormal. In contrast to double-mutant clones, *mer* or *ex* single-mutant retinae showed only a few extra interommatidial cells (Fig. 1g, h), further supporting that *mer* and *ex* can partially substitute for each other. Notably, the *mer;ex* double-mutant phenotypes are remarkably similar to those of *hpo* mutants (Fig. 1i)[6,8–10], but are distinct from the phenotypes of mutations in other growth control genes, such as *TSC1 (Tuberous Sclerosis Complex 1)*, which affects cell size (Fig. 1j, j')[34,35]. Therefore, Mer and Ex, like Hpo, regulate the number of cells in epithelial tissues.

Merlin and Expanded regulate cell-cycle arrest and Cyclin E transcription

The observation that *mer;ex* double-mutant retinae have more interommatidial cells raised two questions: How are these extra cells generated and why are they not eliminated by apoptosis? First, we examined the effects of *mer* and *ex* on cell proliferation by monitoring bromodeoxyuridine (BrdU) incorporation, which marks cells in S-phase. During the third larval stage, a wave of differentiation — the morphogenetic furrow — sweeps across the eye disc from posterior to anterior[32,33]. Cells anterior to the furrow are undifferentiated and divide asynchronously.
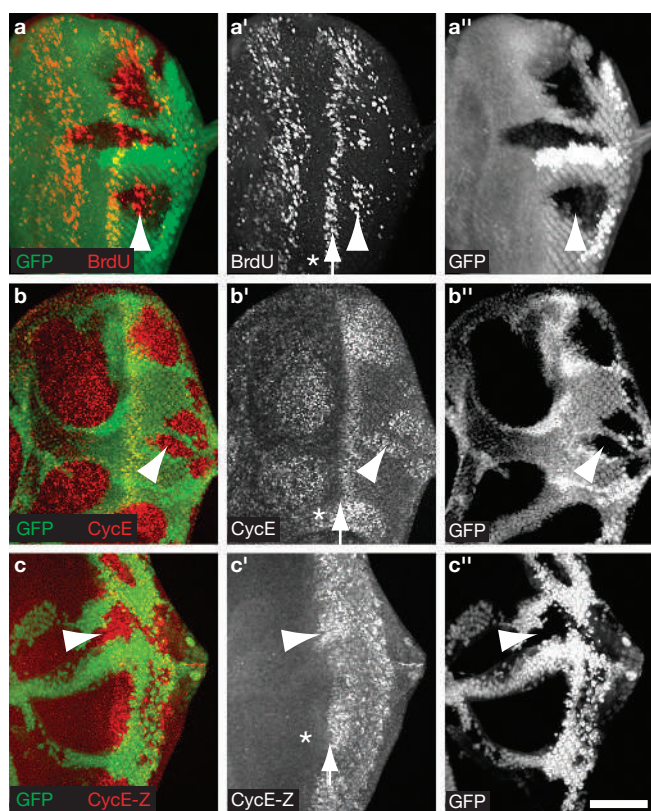
Figure 2 *merlin* and *expanded* regulate cell-cycle arrest and the expression of Cyclin E. All panels show third instar eye imaginal discs containing *mer⁻;ex⁻* double-mutant clones marked by the absence of green fluorescent protein (GFP) expression (green in **a**, **b**, **c**, and grayscale in **a"**, **b"**, **c"**). The disc in (**a-a"**) is labelled for bromodeoxyuridine (BrdU) incorporation (red in **a**, grayscale in **a'**). Wild-type cells arrest in G1 in the morphogenetic furrow (asterisk) and non-differentiating cells go through one synchronous S phase in the second mitotic wave (arrows). *mer⁴;exᵉ¹* mutant cell clones show ectopic cell proliferation posterior to the second mitotic wave (arrowheads). (**b-b"**) *mer⁴;exᵉ¹* mutant cells have increased levels of Cyclin E (CycE) protein (red in **b**, grayscale in **b'**) both anterior and posterior to the morphogenetic furrow (asterisk). (**c-c"**) *mer⁴;exᴬᴾ⁵⁰* mutant cells have elevated levels of *lacZ* expression from the 16.4 lacZ Cyclin E reporter construct (CycE-Z)[37] (red in **c**, grayscale in **c'**). Anterior is to the left for all discs. All alleles shown are null alleles. Scale bar (**c"**), 50 µm.

Behind the furrow, cells either differentiate into photoreceptor cells or undergo one additional round of cell division — referred to as the second mitotic wave — before they differentiate into the remaining photoreceptor, cone, pigment and bristle cells[32,33]. *mer;ex* mutant cells properly synchronized their cell cycles in the furrow (Fig. 2a', asterisk) and progressed through the second mitotic wave (Fig. 2a', arrow). In contrast to wild-type cells, however, cells in *mer;ex* mutant clones displayed ectopic BrdU incorporation after the second mitotic wave (Fig. 2a–a", arrowhead). This ectopic DNA synthesis is followed by cell division, as seen by ectopic phosphorylated histone H3 expression, which marks mitotic chromosomes (not shown). Mutant cells, therefore, failed to arrest the cell cycle after the second mitotic wave and instead continued to proliferate. Cyclin E is a limiting factor for S-phase entry in imaginal-disc cells[36], and we found that it was upregulated in *mer;ex* double-mutant cells (Fig. 2b–b"). This regulation was at the level of transcription, as shown by elevated

expression of a Cyclin E reporter gene[37] (Fig. 2c–c"). The repression of Cyclin E expression is probably an important downstream effect of Mer and Ex that regulates cell proliferation. We conclude that Mer and Ex are required for proper cell-proliferation arrest.
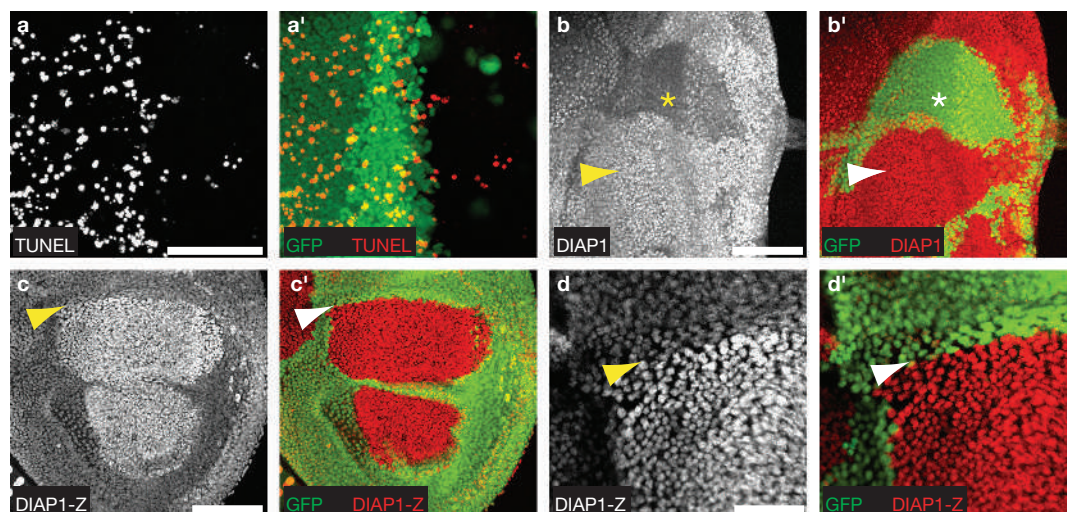
Merlin and Expanded regulate apoptosis

Although the defects in proliferation arrest generate extra cells, this is not sufficient to explain the overgrowth phenotype that is seen in *mer;ex* mutant tissues. This is because developing tissues compensate by eliminating extra cells by apoptosis. We therefore addressed why the extra interommatidial cells in *mer;ex* double-mutant retinae survived, whereas extra cells are eliminated by apoptosis in wild-type retinae[32]. We found that loss of Mer and Ex function suppressed the wave of apoptosis that normally eliminates extra interommatidial cells during the pupal stage (Fig. 3a, a'). We then tested whether Mer and Ex regulate the expression of the anti-apoptotic gene *Drosophila Inhibitor of Apoptosis Protein-1 (diap1)*[38], a target of Hpo signalling[6–12,15]. Indeed, *mer;ex* mutant clones had higher levels of DIAP1 protein (Fig. 3b–b') and cells autonomously upregulated the expression of a *lacZ* enhancer trap[39] insertion in the *diap1* gene (Fig. 3c–d'), indicating that this upregulation was at the level of *diap1* transcription. This extra DIAP1 may protect cells from apoptosis that is induced during development. We conclude that Mer and Ex are required for developmentally induced apoptosis that normally eliminates extra cells. The combination of the defects in cell-cycle exit and apoptosis observed in *mer;ex* clones generates extra cells that then evade apoptosis and produce overgrowths in the adult.

Merlin and Expanded act genetically upstream of Hippo

The phenotypes of *mer;ex* double-mutant tissues are strikingly similar to those of mutations in *hpo, sav* and *wts*. Mutations in all of these genes cause extra interommatidial cells, ectopic BrdU incorporation posterior to the morphogenetic furrow and cell-autonomous elevation of Cyclin E and DIAP1 expression within mutant cells. This combination of phenotypes is not observed for mutations in other known growth control genes, and therefore suggests that Mer and Ex interact with Hpo signalling. In addition, we found that the loss of one copy of *wts* enhanced the imaginal-disc overgrowth phenotypes of hypomorphic mutations in *ex* (see Supplementary Information, Fig. S2a–d), and that loss of one copy of *ex* enhanced the larval lethality caused by hypomorphic *wts* mutations (see Supplementary Information, Fig. S2e). Based on these observations, we hypothesized that Mer and Ex act in Hpo signalling. In the next set of experiments, we tested this hypothesis using overexpression of Ex and Hpo in different mutant backgrounds. We focused on the overexpression of Ex and Hpo because ectopic Mer, Wts or Sav cause only weak phenotypes[11] (and data not shown).

We and others previously reported that overexpression of Hpo hyperactivates Hpo signalling and causes a reduction in cell proliferation and induces apoptosis, phenotypes that are opposite to those of its loss-of function phenotypes (Fig. 4a, a', f, f')[6–8,10]. Similarly, overexpression of Ex during eye development induced apoptosis, reduced cell proliferation and severely disrupted eye development (Fig. 4b, b')[30,31]. These effects are not caused only by induction of apoptosis, because the phenotypes of ectopic Ex or Hpo are not completely rescued when we inhibited apoptosis by coexpression of the caspase inhibitor p35 or DIAP1 (Fig. 4a–c, a'–c'; and data not shown)[8,31]. Although coexpression of p35 with Ex gave only a partial rescue, loss of Hpo function reversed the ectopic Ex

Figure 3 *merlin* and *expanded* are required for developmentally induced apoptosis and regulate DIAP1 expression. All panels show third instar eye imaginal discs containing *mer⁻;ex⁻* double-mutant clones marked by the absence of green fluorescent protein (GFP) expression (green in **a'**, **b'**, **c'**, **d'**). (**a, a'**) Confocal image showing TUNEL labelling to detect dying cells (grayscale in **a**, red in **a'**) of a pupal retina 25 h after pupariation with *mer⁴;ex^{e1}* mutant cells. Scale bar, 50 μm. (**b, b'**) *mer⁴;ex^{e1}* mutant clones upregulated DIAP1 expression (grayscale in **b**, red in **b'**). Arrowheads point to a mutant area and asterisks mark the basal levels of DIAP1 expression in a wild-type area. Scale bar, 50 μm. (**c, c'**) *mer⁴;ex^{AP50}* mutant clones (arrowheads) upregulated the expression of a *lacZ* enhancer trap insertion into the *DIAP1* gene (grayscale in **c**, red in **c'**). Scale bar, 50 μm. (**d, d'**) Close up of a region of the disc in (**c, c'**), showing that upregulation of DIAP1–lacZ (DIAP1-Z) in *mer;ex* mutant clones was cell autonomous (arrowhead points to the clone boundary). Anterior is to the left for all discs. All alleles used are null alleles. Scale bar, 20 μm.

phenotypes. Therefore, the loss of Hpo function reversed the loss of interommatidial cells, the disruption of ommatidial morphology and the reduction in eye size caused by overexpressed Ex. As a result, *hpo* mutant eyes that overexpressed Ex showed the same phenotype as *hpo* mutant eyes (Fig. 4d, d', e, e'). Therefore, Ex requires Hpo for its function. On the other hand, the phenotypes caused by Hpo overexpression were not suppressed by loss of *ex* or in *mer;ex* double-mutant clones (Fig. 4f, f', g, g', k, k'). Together, these results indicate that Hpo acts downstream of Ex.

In support of this model, the effects of both overexpressed Ex and overexpressed Hpo were suppressed by loss of *wts*, which is known to act downstream of Hpo[6,8,10,17] (Fig. 4i–j', l, l'). Furthermore, loss of one copy of the *wts* gene partially suppressed the phenotypes of ectopic Ex, further indicating that Wts acts downstream of Ex and that Wts is required for the effects of Ex (Fig. 5a–c, a'–c').

In addition to the defects in morphology and interommatidial cell number caused by the overexpression of Ex and Hpo, we used the induction of apoptosis in third instar eye discs as a readout. Overexpression of Ex or Hpo posterior to the morphogenetic furrow induced a stripe of dying cells. The cell death induced by overexpressed Ex was suppressed in *hpo* or *wts* mutant clones, but loss of *mer* and *ex* had no effect on Hpo-induced cell death (Fig. 5d–f). Overexpressed Ex therefore requires Hpo to induce cell death but not *vice versa*. We conclude that Mer and Ex act genetically upstream of Hpo and Wts.

Merlin and Expanded regulate Warts phosphorylation and Yorkie activity

Having established that Mer and Ex act upstream of Hpo genetically, we investigated whether Mer and Ex affected Wts phosphorylation. Previous analyses showed that Hpo induces phosphorylation of Wts, and that the phosphorylation status of Wts is a readout for Hpo pathway activity[8]. In S2 cells, overexpression of Hpo and Sav

induces phosphorylation of Wts, which is visualized as a shift in Wts mobility (Fig. 6a)[8]. We found that coexpression of Mer and Ex led to a further shift in Wts mobility (Fig. 6a). This shift was due to protein phosphorylation, as it could be reversed by phosphatase treatment (Fig. 6b). In addition, overexpression of Mer and Ex were sufficient to induce phosphorylation of Wts without overexpression of Hpo and Sav (Fig. 6a). Possibly, endogenous Hpo and Sav are sufficient to mediate this effect. Whereas coexpression of Mer and Ex led to a complete shift in Wts mobility, overexpression of Mer without Ex led to an incomplete shift of Wts, and overexpression of Ex alone had no effect on Wts mobility (Fig. 6a). Therefore, Mer and Ex act synergistically to induce phoshorylation of Wts in cultured cells. Although Mer and Ex affect the phosphorylation status of Wts, they did not immunoprecipitate with Hpo, Sav or Wts *in vitro* or *in vivo* (data not shown). Thus, Mer and Ex may only transiently associate with Hpo-containing complexes, or other, as yet unidentified, components may link Mer and Ex to Hpo and Wts.

Huang *et al.* recently identified the transcriptional coactivator Yorkie (Yki) as a downstream target of Wts[16]. Activated Wts phosphorylates Yki and suppresses its transcriptional activator function[16]. In S2 cells a Gal4-DNA-binding domain–Yki fusion protein can activate a UAS–luciferase reporter construct and this activity is suppressed by coexpression of Hpo, Sav and Wts[16]. We found that Ex and Mer also downregulated the activity of Yki, and reduced it to a level similar to that achieved by expression of Hpo, Sav and Wts (Fig. 6c). Coexpression of Ex and Mer with Hpo, Sav and Wts led to an even stronger reduction of Yki activity (Fig. 6c). This effect is specific because the expression of Mer, Ex, Hpo, Sav and Wts did not affect the activity of full-length Gal4 (Fig. 6d). These experiments show that Mer and Ex regulate the activity of Yki, which is currently the most downstream component known in the Hpo signal-transduction pathway.
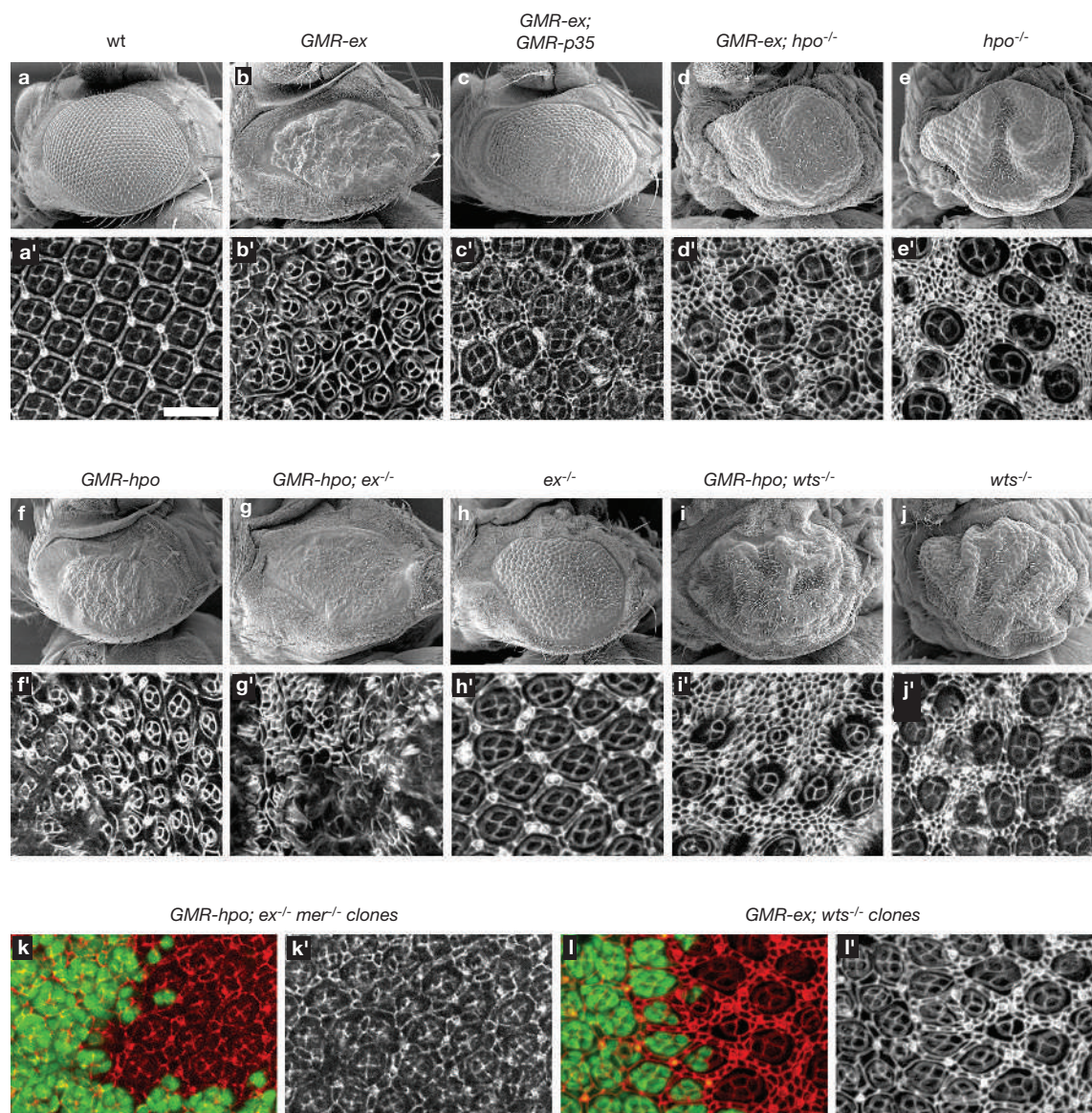
Figure 4 Expanded acts genetically upstream of Hippo. Panels (**a-j**) show scanning electron microscopy images of adult eyes and panels (**a'-j'**) display mid-pupal retinae stained for anti-Discs large (Dlg). Panels (**k-l'**) show pupal retinae carrying *mer;ex* (**k-k'**) and *wts* (**l-l'**) mutant clones that are marked by the absence of green fluorescent protein expression (green in **k, l**). Retinae are stained for Dlg (red in **k, l** and gray in **k'**, **l'**). The genotypes of the animals are indicated above the panels. *GMR* refers to *GMR–gal4*-driven overexpression of the indicated transgenes (*UAS–ex, UAS–hpo* or *UAS–p35*). *GMR–gal4* drives expression of these transgenes in the developing eye posterior to the morphogenetic furrow. Mutant heads were generated via *eyFLP*-mediated mitotic recombination to generate heads that were entirely mutant for the indicated mutations. For experiments shown in panels (**d, d', e, e', g', i, j, j'** and **k–l'**), the mutant chromosome was flipped against a chromosome marked with *ubiGFP*. (**h'**) shows an *ex^BQ* homozygous mutant retina and, in (**g, h, i'**), *ex* and *wts* mutant chromosomes were flipped against chromosomes carrying a *cell lethal* mutation. The null mutations *mer^4*, *hpo^{42-47}*, *ex^BQ* and *wts^{x1}* were used. Scale bar (**a'**), 20 µm.

Hippo signalling regulates the expression of Merlin and Expanded

It was previously observed that the levels of Ex protein are elevated in *mer* mutant cells[27]. We therefore tested whether this negative regulation was due to Hpo signalling. Indeed, we found that the expression of *mer* and *ex* is tightly regulated by Hpo signalling. Cells that are mutant for *hpo, sav* or *wts* had highly elevated levels of both Ex and Mer proteins (Fig. 7a–b', and see Supplementary Information, Fig. S3). The subcellular localization of Mer and Ex to the apical-lateral membrane was, however, not affected (Fig. 7c–d'). The upregulation was due to derepressed transcription, because we detected elevated levels of *ex* transcripts in discs with *hpo* or *wts* mutant clones (Fig. 7e–h, and data not shown), and because the expression of a *lacZ* enhancer trap insertion into the *ex* locus, which reports the transcription of the *ex* locus, was strongly induced in *wts* and *sav* mutant clones (Fig. 7i–i'; and data not shown). Significantly, Mer and Ex were derepressed in *hpo, wts* or *sav* mutant clones in different discs independently of the developmental stage of the discs or the position of the clones. The negative-feedback regulation of *mer* and *ex* expression by Hpo
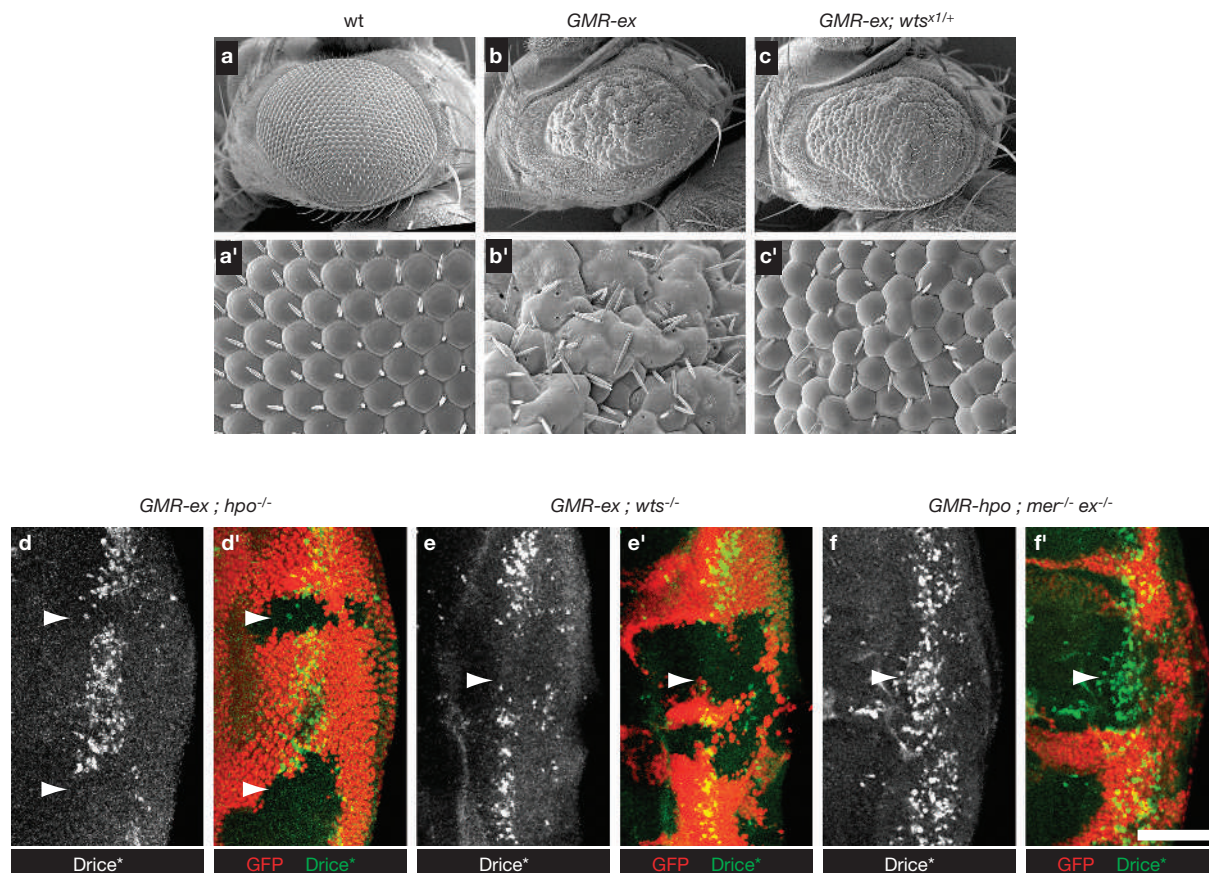
Figure 5 Merlin and Expanded act upstream of Hippo and Warts. Panels (**a-c**) show scanning electron microscopy images of the eyes of adult male flies. The genotypes of the animals are indicated above the panels. *GMR–ex* refers to *GMR–gal4*-driven overexpression of a *UAS–ex* transgene. wt, wild type. (**a'-c'**) show higher magnification images of the panels above them. Removal of one copy of *wts* partially suppresses the reduced and rough-eye phenotype caused by overexpression of Ex. (**d-f'**) Anti-Drice labelling of third instar eye imaginal discs with indicated genotypes. (**d, d'**) *hpo*[42–47] or (**e, e'**) *wts*[x1] mutant clones suppress cell death induced by overexpressed Ex, whereas (**f, f'**) *mer*[4];*ex*[BQ] double-mutant clones do not suppress ectopic Hpo-induced cell death. Panels (**d, e, f**) show Drice labelling only. Mutant areas are marked by the absence of green fluorescent protein (GFP; red in **d', e', f'**). Arrowheads point to mutant areas and anterior is to the left for all discs. Scale bar (**f'**), 50 μm.
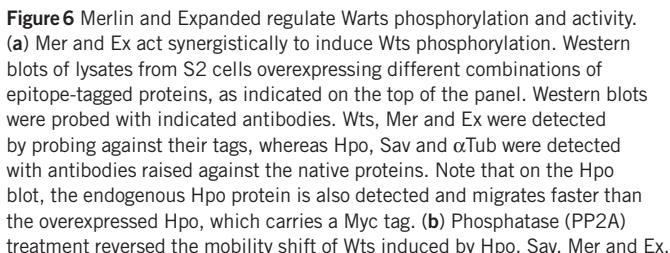
signalling is therefore a general effect. Indeed, it is not uncommon that the expression of signalling-pathway components is regulated in a feedback loop by the pathway itself[40]. This strong and tissue-independent regulation of *mer* and *ex* expression by Hpo signalling therefore supports the idea that they act together in a signalling pathway and may provide an important feedback mechanism to keep Hpo signalling in a steady state.

The identification of *mer* and *ex* as transcriptional target genes of Hpo signalling leads to a model in which both Mer and Ex regulate the expression of themselves. Indeed, we corroborated that the levels of Ex are elevated in *mer* mutant cells (Fig. 7k, k')[27] and we found that Mer is upregulated in *ex* mutant clones (Fig. 7l, l'). In addition, we found elevated levels of Ex in cells mutant for a strong hypomorphic *ex* allele, *ex*[AP49], which encodes a protein that is truncated after the FERM domain and that is still recognized by the anti-Ex antibodies (Fig. 7m, m'; and see Supplementary Information, Fig. S1). Moreover, expression of *lacZ* from the *ex* enhancer trap insertion was strongly induced in *mer;ex* double-mutant cells (Fig. 7j, j'). Therefore, like other components of the Hpo signalling pathway, Mer and Ex negatively regulate the expression of *mer* and *ex*, thereby supporting the model in which Mer and Ex act in Hpo signalling.

Hippo hyperactivation rescues transcriptional defects in *expanded* mutant cells

Once we identified *mer* and *ex* as ubiquitous targets of Hpo signalling, we used the expression of *mer* and *ex* as readouts for Hpo pathway activity in epistasis experiments. We thus tested whether hyperactivation of Hpo was sufficient to suppress the induction of Ex expression in *ex*[AP49] mutant cell clones. To do this experiment, we used the GMR–*gal4* driver to overexpress Hpo specifically posterior the morphogenetic furrow. As expected, in such discs, *ex*[AP49] mutant clones still upregulated Ex anterior to the furrow where Hpo was not overexpressed (Fig. 7m, m', asterisk). However, the hyperactivation of Hpo posterior to the furrow suppressed the upregulation of Ex in *ex*[AP49] mutant clones (Fig. 7n, n', arrowhead). Hpo activation is therefore sufficient to rescue transcriptional defects in *ex* mutant cells.

Finally, we tested whether ectopic Hpo could suppress endogenous Ex. Clones of cells overexpressing Hpo alone or in combination with DIAP1 (to suppress cell death caused by overexpression of Hpo) showed reduced levels of Ex (Fig. 7o–o'''; and data not shown). These cells, however, showed normal levels of Dlg at the apicolateral junctions indicating that loss of Ex was not due to general defects in cell architecture. The results indicate that in wild-type cells, the activity of the Hpo pathway is in a steady state and can be positively or negatively modulated.

a

Transfected with

Wts	W	W	W	W	W	W	W	W
Hpo	–	H	H	–	–	–	–	–
Sav	–	S	S	–	–	–	–	–
Ex	–	–	E	–	E	E	–	–
Mer	–	–	M	–	M	–	M	–

α-V5 (Wts)

α-Hpo

α-Sav

α-HA (Ex)

α-HA (Mer)

α–αTub

1 2 3 4 5 6 7 8

b

Wts	W	W	W	W	W	W	W	W	W
Hpo	H	H	–	–	–	–	–	–	–
Sav	S	S	–	S	S	–	–	–	–
Ex	–	–	–	E	E	–	–	E	E
Mer	–	–	M	M	M	–	M	M	M

PP2A   +   + +   +

1 2 3 4 5 6 7 8 9

α-V5 (Wts) western blot

c

Fold activation

Gal4DBD	+	–	–	–	–	–	–	–	–
Gal4DBD-Yki	–	+	+	+	+	+	+	+	+
Hpo Sav	–	–	HS	HS	–	–	–	–	–
Wts	–	–	W	W	–	–	–	W	W
Ex	–	–	–	E	E	E	–	–	E
Mer	–	–	–	M	M	–	M	–	M

d

Fold activation

Gal4DBD	+	–	–
Gal4-FL	–	+	+
Hpo Sav	–	–	HS
Wts	–	–	W
Ex	–	–	E
Mer	–	–	M

Figure 6 Merlin and Expanded regulate Warts phosphorylation and activity. (**a**) Mer and Ex act synergistically to induce Wts phosphorylation. Western blots of lysates from S2 cells overexpressing different combinations of epitope-tagged proteins, as indicated on the top of the panel. Western blots were probed with indicated antibodies. Wts, Mer and Ex were detected by probing against their tags, whereas Hpo, Sav and αTub were detected with antibodies raised against the native proteins. Note that on the Hpo blot, the endogenous Hpo protein is also detected and migrates faster than the overexpressed Hpo, which carries a Myc tag. (**b**) Phosphatase (PP2A) treatment reversed the mobility shift of Wts induced by Hpo, Sav, Mer and Ex.

Note low phoshorylation levels of overexpressed Wts, which is also diminished by phosphatase treatment (lanes 6 vs. 7). (**c**) The coactivator activity of Yki is negatively regulated by Mer and Ex. S2 cells were transfected with UAS–luc (Gal4-responsive luciferase reporter) plasmid along with plasmids expressing the Gal4 DNA-binding domain-Yki fusion protein and plasmids expressing Hpo, Sav, Wts, Ex and Mer as indicated. Luciferase activities normalized to that induced by the Gal4 DNA-binding domain (Gal4DBD) alone are plotted. Error bars represent standard deviations from three independent transfections. (**d**) The transcriptional activator function of the full-length Gal4 (Gal4-FL) is not affected by expression of Hpo, Sav, Wts, Ex and Mer.

DISCUSSION

Our data functionally link the tumour-suppressor functions of Mer and Ex with Hpo signalling. We propose a model in which Mer and Ex act together to transduce a growth-regulatory signal from an unknown receptor to Hpo and Wts, which in turn regulate the expression of target genes to promote proliferation arrest and apoptosis (Fig. 8). We provided several lines of evidence that place the activities of Mer and Ex upstream of Hpo and Wts. First, the phenotypes induced by overexpression of Ex in imaginal discs are reversed by removing either Hpo or Wts, whereas Hpo-induced phenotypes do not require the activity of Mer and Ex. Second, hyperactivation of Hpo is sufficient to rescue the transcriptional defects of *ex* mutant cells. Third, Mer and Ex act synergistically to induce phosphorylation of Wts. Fourth, Mer and Ex regulate the activity of Yki, a Wts target, in a cell-culture-based assay. Together, these data support a model in which Mer and Ex act upstream and through Hpo signalling to perform their tumour-suppressor functions in *Drosophila* (Fig. 8).

Does Mer act through Hpo signalling in vertebrates? All currently known components of the Hpo signalling pathway in *Drosophila* are highly conserved in vertebrates, where they have been implicated in regulating cell proliferation and apoptosis. Mice and humans have two Wts homologues, Lats1 and Lats2, and mice deficient for Lats1 develop soft-tissue sarcomas and ovarian tumours[41–43]. Therefore, Lats1 acts as a tumour-suppressor gene in vertebrates. Sav may also have an effect on tumorigenesis, because the human orthologue of Sav, Sav1, is mutated in cancer cell lines[11]. Vertebrates have two Hpo homologues, Mst1 and Mst2 and, as in *Drosophila*, they promote apoptosis and regulate cell-cycle exit[44]. Therefore, the Hippo signalling pathway regulates proliferation and apoptosis in vertebrates.

Human Mer has been shown to physically interact with several other proteins *in vitro* and in cell culture cells[20,24,25]. However, how these interactions contribute to the tumour-suppressor function of Mer is unclear[20,24,25]. So far, none of these components have been linked to Mst1/2 signalling, but the
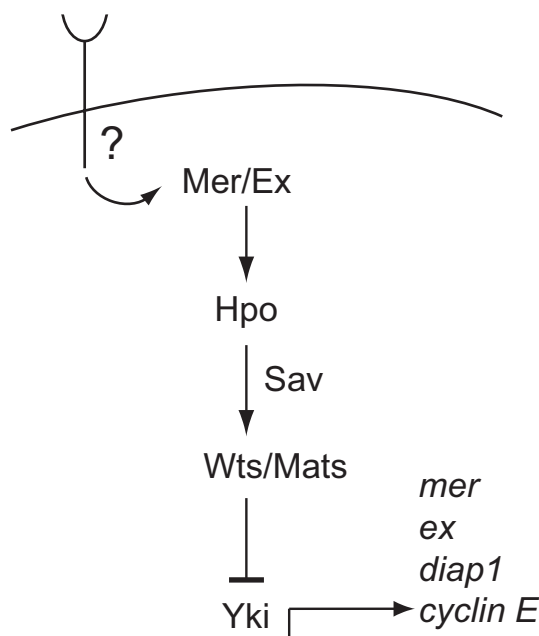
Hpo signalling pathway has only recently been discovered. Notably, using BLAST searches, we found two vertebrate homologues of Ex we called Ex1 and Ex2 (see Supplementary Information, Fig. S1). Therefore, all known components of the Hpo signalling pathway are conserved in vertebrates. Importantly, not only the genes but also the mechanisms of signal transduction seem to be conserved between *Drosophila* and vertebrates. Vertebrate Lats1 and Mst2 can rescue the lethality and overgrowth phenotypes of *wts* and *hpo* mutants[8,43] and Mst1/2 bind to Sav1 and phosphorylate and activate Lats1/2 (ref. 17). Due to this apparent conservation of the components and mechanisms that operate downstream of Mer and Ex in *Drosophila* and in vertebrates, Mer and the two Ex homologues may function as tumour-suppressor genes through the Hpo homologues Mst1/2. Our data that links Mer to Hpo signalling may therefore have important implications for the study and treatment of neurofibromatosis and other cancers. □

METHODS

Drosophila **stocks.** Mutant clones were induced using the FLP/FRT system[45,46]. To generate of marked double-mutant clones, we recombined *p{w⁺ mer⁺}[27]* with a *FRT40A ubiGFP* chromosome. Flies with *mer;ex* double-mutant clones had the following genotype: *y w mer⁴FRT19A/ Y; FRT40A ex^e1/FRT40A p{w⁺ mer⁺} ubiGFP; hsFLP MKRS/+*, except for those seen in Figs 2c–c" and 3c–d', in which we used *ex^AP50* because *ex^e1* is an enhancer trap itself. After mitotic recombination took place, non-green-fluorescent-protein (GFP)-expressing cells were homozygous mutant for *ex* and lacked the *mer* rescue construct; they were, therefore, double mutant for *mer* and *ex*. To generate *ex, mer, hpo, wts, sav* or *TSC1* mutant clones, the following alleles were flipped against corresponding *ubiGFP* marked *FRT* chromosomes: *ex^e1* (null)[31], *ex^BQ* (null; see Supplementary Information, Fig. S1), *ex^AP49* (hypomorph, see Supplementary Information, Fig. S1), *mer⁴* (null)[29], *hpo^42–47* (null)[8], *wts^x1* (null)[14], *wts^P2* (hypomorph)[13], *sav^shrp1* (null)[1,2] and *TSC1^Q69* (ref. 6). Overexpression was achieved using the UAS–GAL4 system[47] and the following stocks: *UAS–ex* (ref. 30), *UAS–hpo* (ref. 6), *UAS–p35* (ref. 38) and *GMR–gal4*. Overexpression clones of Hpo and DIAP1 were induced using the flp-out technique[48] and the following stocks: *ywhsflp; act<y+<Gal4, UASGFP^nls/CyO* and *UAS–DIAP1* (obtained from B.Hay, Caltech, USA). Other stocks used were: *ex^697/CyO* (ref. 31), *DIAP1–lacZ* (ref. 38) and *CycE–lacZ* (16.4 construct[37]).

Figure 7 Hippo signalling regulates Merlin and Expanded expression and ectopic Hippo is sufficient to rescue *expanded* mutant phenotypes. Mer and Ex protein levels are elevated in clones of Hpo pathway mutants, whereas their subcellular localization is not affected. (**a-b'**) hpo^{42-47} mutant clones in eye-antennal imaginal discs, marked by the absence of green fluorescent protein (GFP; green in **a'** and **b'**), have increased levels of Ex (grayscale in **a**, red in **a'**) and Mer (grayscale in **b**, red in **b'**) proteins. Upregulation of Ex and Mer is particularly prominent in the morphogenetic furrow (arrows). Arrowheads point to mutant clones. Scale bar (a), 50 μm. (**c-d'**) Z-sections through wing imaginal discs with wts^{x1} mutant clones that are marked by the absence of GFP (green in **c'** and **d'**). Localization of Ex (grayscale in **c**, red in **c'**) and Mer (grayscale in **d**, red in **d'**) is not altered in wts^{x1} mutant cells, whereas protein levels are elevated. (**e**) Third instar eye imaginal disc bearing wts^{P2} mutant clones marked by the absence of GFP. (**f**) *ex* mRNA expression in a wild-type eye-antennal imaginal disc detected by *in situ* hybridization. (**g, h**) Increased levels of *ex* mRNA are detected in discs with wts^{P2} mutant clones. Arrows point to the morphogenetic furrow and arrowheads mark patches of upregulated *ex* expression. (**i-j'**) Eye imaginal discs bearing wts^{x1} or $mer^4;ex^{e1}$ mutant clones and showing the expression of β-Gal driven by an enhancer trap insertion into

the *ex* gene. Grayscale in (**i, j**) shows β-Gal expression only. Scale bar (j), 50 μm. (**k, k'**) Higher levels of Ex protein (grayscale in **k**, red in **k'**) are present in mer^4 mutant clones in a wing disc marked by the absence of GFP (arrowhead). (**l, l'**) ex^{e1} mutant clones (GFP-negative) upregulate Mer expression (grayscale in **l**, red in **l'**). (**m, m'**) ex^{AP49} mutant clones (GFP-negative, arrowheads) have elevated levels of Ex protein (grayscale in **m**, red in **m'**) both anterior and posterior to the morphogenetic furrow (arrow). (**n, n'**) Upregulation of Ex (grayscale in **n**, red in **n'**) in ex^{AP49} mutant clones is suppressed by ectopic expression of Hpo behind the morphogenetic furrow. Asterisk marks Ex upregulation anterior to the furrow. Arrowheads point to the mutant clones with repressed Expanded expression. Hpo overexpression was driven by *GMR–gal4*, which drives expression posterior, but not anterior to the morphogenetic furrow (arrow). White bar marks the region of *GMR–hpo* expression. Ex overexpression is therefore only suppressed posterior to the furrow, where Hpo is expressed. (**o-o'''**) Wing imaginal disc with clones overexpressing Hpo and DIAP1 downregulate the expression of Ex (gray in **o'** and red in **o** and **o'''**), although do no affect Dlg (blue in **o**, gray in **o''**). Clones overexpressing Hpo and DIAP1 are marked by the coexpression of GFP (green in **o** and **o'''**). Scale bar (**o'''**), 100 μm. Anterior is to the left for all discs.

Figure 8 Model of the Hpo signalling pathway. Mer and Ex act upstream of Hpo and regulate the expression of *mer, ex, diap1* and *cyclin E* transcription. Mer/Ex may transduce the signal of an, as yet unknown, growth-regulating cell-surface receptor.

Scanning electron microscopy, immunohistochemistry, *in-situ* hybridization and cell-death assays. Scanning electron microscopy (SEM) of adult flies was carried out following the hexamehyldisilazane (HMDS) method[12]. Antibody stainings of imaginal discs were performed as described previously[12]. The following antibodies were used (dilutions and source in parentheses): mouse α-Dlg (1:300; DSHB); rabbit α-Drice (1:2000; B. Hay); guinea-pig α-Mer (1:4000; R. Fehon, Duke University, USA); guinea-pig α-Ex (1:2000; R. Fehon); rabbit α-Ex (1:1500; A. Laughon, University of Wisconsin, USA); mouse α-BrdU (Becton-Dickinson, Franklin Lakes, USA; 1:50); mouse α-DIAP-1 (1:200; B. Hay); and mouse α-CycE (1:40). Secondary antibodies were donkey Fab fragments from Jackson Immuno Research (West Grove, PA). BrdU incorporation was carried out as described previously[12], by incorporating BrdU for 1 h[12]. For the *in situ* hybridization to detect *expanded* transcripts, the *Drosophila* cDNA clone UG24F8 was used as a template to generate DIG-labelled RNA probes (Roche, Indianapolis, IN). TUNEL labelling was performed on imaginal discs and pupal retinae using an *in situ* cell-death detection kit (Roche)[6].

Cell culture. N-terminal tagged HA-Ex and HA-Mer were constructed using the pAc5.1 vector. All other constructs were gifts from Duojia Pan, Johns Hopkins University, USA[8,16]. *Drosophila* S2 cells, cultured in Schneiders's medium containing 10% fetal bovine serum and antibiotics were transiently transfected using Cellfectin (Invitrogen, Carlsbad, CA) and collected 48 h after transfection. Lysis was performed in RIPA Buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin and phosphatase inhibitors (Roche)), and Western blots were performed according to standard protocols. Antibodies used were: α-V5 (Invitrogen); α-HA (CRP, Cumberland, VA); and α-Hpo, α-Sav and α-αTub (Sigma, St Louis, MO). α-Hpo and α-Sav antibodies were raised in guinea pigs against native His-tagged proteins.

For phosphatase treatments, cells were lysed in IP buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% NP40, 1 mM PMSF, 5 μg ml^{-1} aprotinin, 5 μg ml^{-1} leupeptin) and V5-Wts proteins were immunoprecipitated. Beads were washed three times in phosphatase treatment buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.5 mM MgCl₂, 0.5 mM MnCl₂, 0.5 mM CaCl₂) and 0.3 units of PP2A (Upstate, Charlottesville, VA) were added in a total volume of 75 μl, followed by 30 min incubation at 37 °C. The reaction was terminated by addition of an equal volume of 2× SDS sample buffer.

Luciferase reporter gene assays were performed by transfecting 20 ng of Yki–Gal4 or Gal4–FL plasmids with 3 ng of UAS–luc plasmid in triplicates, with or without plasmids expressing Ex, Mer, Hpo, Sav or Wts in 24-well plates. Luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI) and a 20/20ⁿ Luminometry System with Single Auto-Injector (Promega).

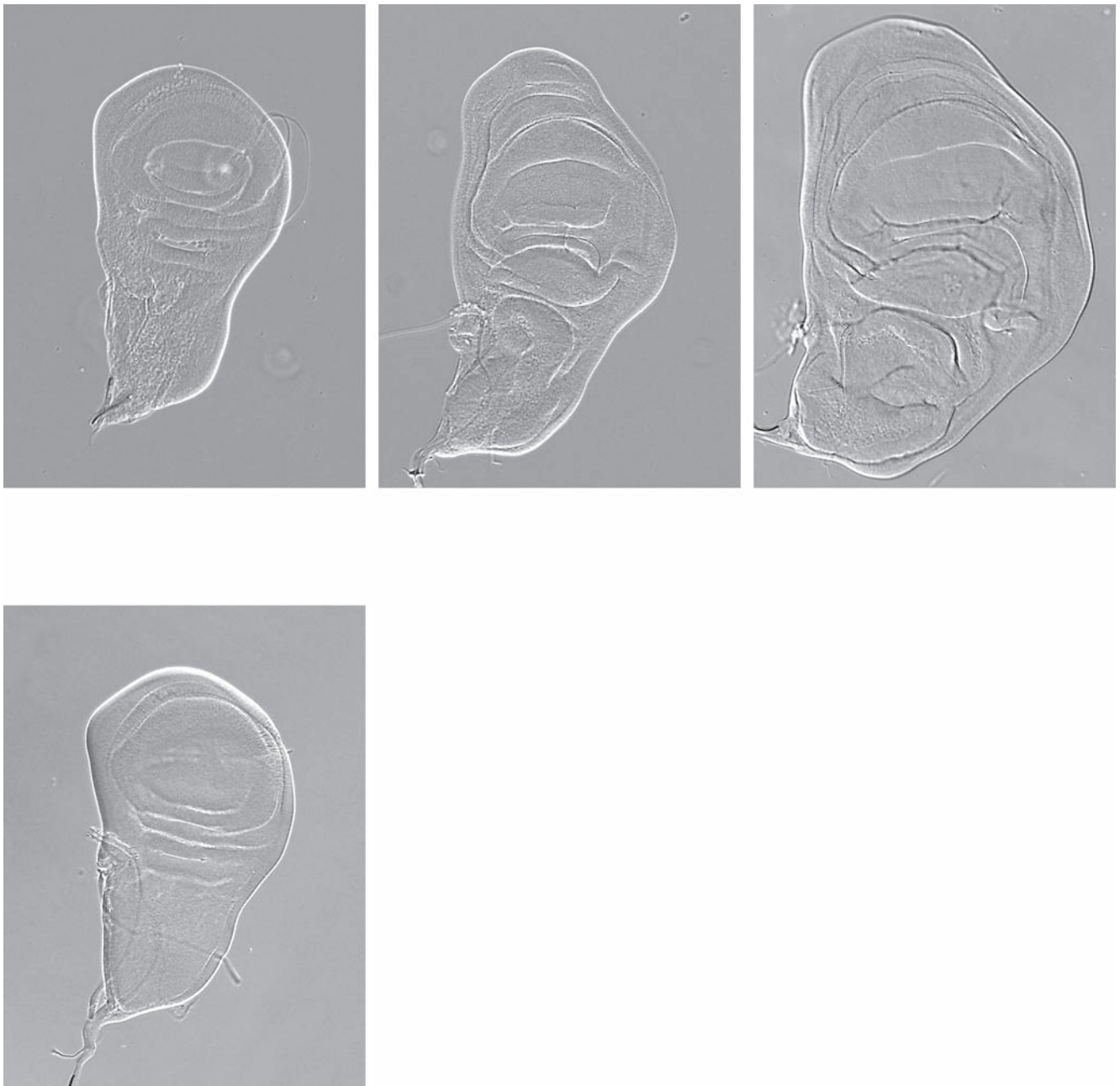*Note: Supplementary Information is available on the Nature Cell Biology website.*

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a

b

Figure S1 Expanded mutant alleles and human Expanded homologs.
(a) Schematic representation of the protein structures of the *Drosophila melanogaster, Anaphilis gambiae*, and *Homo sapiens* Ex homologues. Regions of conserved domains are shaded. Arrows indicate the positions of the mutations in the four newly isolated *ex* alleles. The mutations in alleles *ex^BQ^*, *ex^BB1172^*, and *ex^AP49^* result in premature STOP codons (asterisk) at amino

acid positions 32, 223 and 986, respectively. *ex^AP50^* has a rearrangement which result in a frameshift. The Ex^AP50^ protein is truncated after amino acid 129 followed by 34 unrelated aminoacids with no homology to sequences in GenBank. (b) Sequence alignment of *Drosophila* Ex (Dm), human Ex1 and Ex2 (Hs1, Hs2), and mosquito Ex (Ag). Identical residues are on black background and similar residues are shaded. The FERM domains are boxed.

Figure S2 Genetic interaction between mutations in *expanded* and *warts*. (a-d) Phase-contrast images of wing imaginal discs of third instar wandering larvae (141-157 hours old after egg laying, raised at 25°C) with the following genotypes (a) $ex^{AP49/+}$; $wts^{x1/+}$, (b) ex^{AP49} homozygote, (c) $ex^{AP49/AP49}$; $wts^{x1/+}$ (d) wild-type. Heterozygosity for both *ex* and *wts* does not cause overgrowth. However, heterozygosity for *wts* enhances the overgrowth phenotype of ex^{AP49} homozygous mutant wing imaginal discs. All images were taken at the same magnification. (e) Heterozygosity for ex^{697}, a weak hypomorph, enhances the larval lethality of wts^{P2} homozygotes. The graph shows the percentage of animals with the indicated genotypes pupating compared to the number that would be expected if they had wild-type viability. These numbers were calculated by counting the number of pupae with the indicated genotypes and comparing it to the number of pupae carrying a balancer chromosome in the stocks $wts^{P2}/$ TM6B and $ex^{697}/$ CyO GFP; $wts^{P2}/$ TM6B raised at 25 °C.
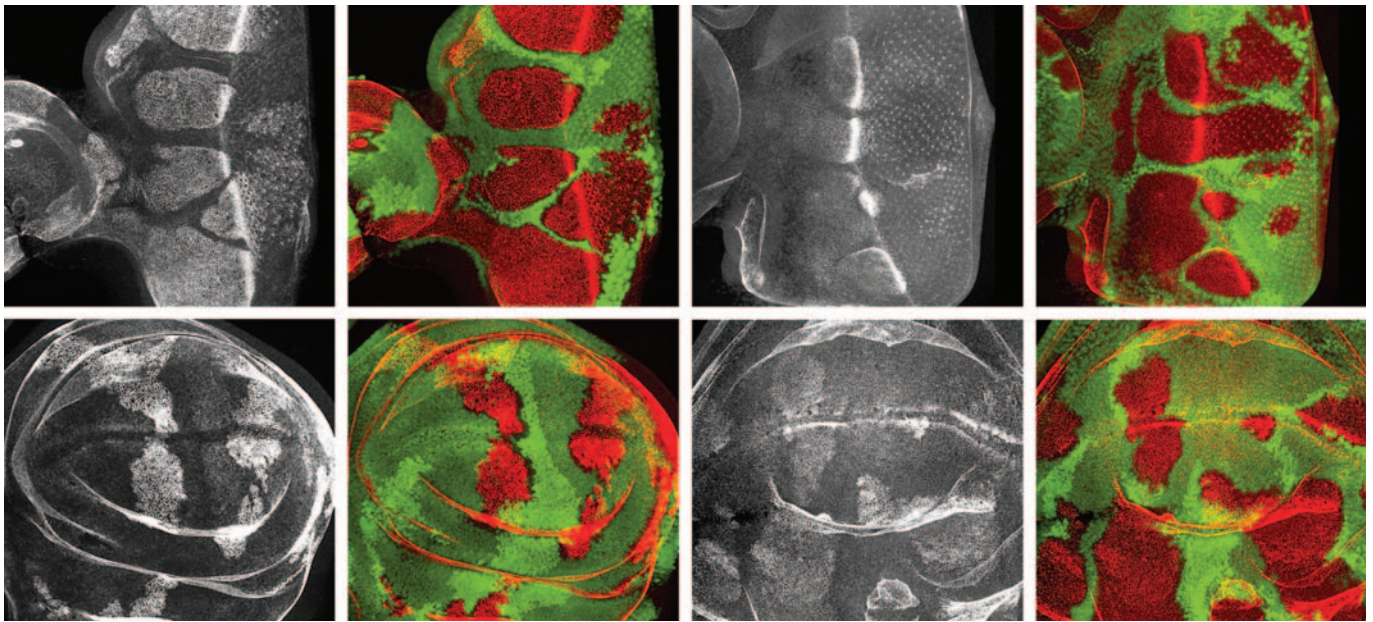
Figure S3 Hippo signalling regulates Merlin and Expanded expression in a tissue independent manner. (a-b') *wts^x1* mutant clones in eye-antennal imaginal discs marked by the absence of GFP (green in a' and b'), have increased levels of Ex (grayscale in a, red in a') and Mer (grayscale in b, red in b') proteins. Upregulation of Ex and Mer is especially prominent in the morphogenetic furrow. (c, c') Higher levels of Ex protein (grayscale in c, red in c') is present in *sav^shrp1* mutant clones marked by the absence of GFP in a third instar wing imaginal disc. (d, d') *wts^x1* mutant clones (GFP negative) in a third instar wing imaginal disc upregulate Mer expression (grayscale in d, red in d').