**Rabaptin-5 and Rabex-5 are neoplastic tumour suppressor genes that interact to modulate Rab5 dynamics in Drosophila melanogaster**

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**Abstract**

Endocytosis plays an important role in the regulation of tumour growth and metastasis. In *Drosophila*, a number of endocytic neoplastic tumour suppressor genes have been identified that when mutated cause epithelial disruption and over-proliferation. Here we characterise the *Drosophila* homologue of the Rab5 effector Rabaptin-5, and show that it is a novel neoplastic tumour suppressor. Its ability to bind Rab5 and modulate early endosomal dynamics is conserved in *Drosophila*, as is its interaction with the Rab5 GEF Rabex5, for which we also demonstrate neoplastic tumour suppressor characteristics. Surprisingly, we do not observe disruption of apico-basal polarity in *Rabaptin-5* and *Rabex-5* mutant tissues; instead the tumour phenotype is associated with upregulation of Jun N-terminal Kinase (JNK) and Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signalling.

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**Introduction**

The endocytic pathway influences many developmental processes including signalling, tissue morphogenesis and cell polarity. Although the endocytic process has been described in great detail in the mammalian cell culture, *in vivo* analysis of its specific developmental roles has been complicated by the essential and constitutive requirement for endocytosis in most cell types, and there remains much to be understood.

In *Drosophila*, many endocytic components have been found to function as neoplastic tumour suppressor genes (reviewed in Vaccari and Bilder (2009)). Loss of function mutations in these genes cause epithelial tissues to over-proliferate, differentiation to be lost and normal tissue architecture including apico-basal polarity, to be disrupted (Herz et al., 2009, 2006; Lu and Bilder, 2005; Menut et al., 2007; Moberg et al., 2005; Morrison et al., 2008; Rodahl et al., 2009; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2009).

Endocytic genes can be grouped into two categories with slightly different tumour phenotypes, according to whether the gene acts early or late in the endocytic pathway. Endocytic tumour suppressor genes such as Rab5, the syntaxin Avalanche (Avl) and the Rab5 effector Rabenosyn-5 (Rbsn-5), act at the level of early endosome fusion (Gorvel et al., 1991; Lu and Bilder, 2005; Morrison et al., 2008; Nielsen et al., 2000). Despite the tumour phenotype of tissue wholly mutant for these genes, clones of mutant cells in an otherwise wild-type background do not over-grow and instead tend to become out-competed by wild-type cells and eliminated from the tissue (Lu and Bilder, 2005). Work in other tumour suppressor mutants in which the phenomenon was first observed, indicates that this probably occurs through a JNK-dependent apoptotic response, which after clone elimination allows tissues to resume their wild-type pattern of growth and development (Agrawal et al., 1995; Brumby and Richardson, 2003; Igaki et al., 2006, 2009; Ohsawa et al., 2011; Uhlirova et al., 2005). However, a second group of tumour suppressor genes, which act later in the endocytic pathway, exhibit a non-autonomous tumour phenotype whereby mutant clones have the ability to promote growth in adjacent wild-type tissue (Herz et al., 2006, 2009; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2009; reviewed in Herz and Bergmann (2009)). These encode some of the components of the ESCRT (endosomal sorting complexes required for transport) complex, which functions in the formation of multivesicular bodies (MVBs, reviewed in Jouvenet (2012)). The difference between these two types of endocytic tumour seems to be due to their differential effects on Notch (N) signalling, caused by ectopic accumulation of N protein at different points of the endocytic pathway, depending upon where the endocytic block occurs (Herz et al., 2006, 2009; Lu and Bilder, 2005; Moberg et al., 2005; Morrison et al., 2008;
Materials and methods

Drosophila stocks and genetics

The following Drosophila stocks were used: From Bloomington stock centre (described in FlyBase): MS1096-GAL4, 459.2-GAL4, ptc-GAL4, pmr-GAL4, UAS-Drk2, cnwsp, P(fy \( \Delta (2-3) \)), Ubx-FLP, FRT42D P[arm-lacZ], P[arm-lacZ] FRT80B. P[Epy2]CG4030EY, P[arm-lacZ] CG4030Del1 (a P-element insertion in Rbpn5 3’UTR), Df(2R)F36 (a deficiency uncovering Rbpn5) and Df(3L)BCS250 (a deficiency uncovering Rabs-5). P(911194)v26367 and P(914133)v6329 are RNAi lines (VDRC). 10xSTAT-GFP (Bach et al., 2007), Gbe-Su(H)-lacZ (Furriol and Bray, 2001) and puc400 (Martin-Blanco et al., 1998) are reporter lines (gifts from Martin Zeidler). ex-lacZ is a reporter line (a gift from Barry Thompson and Boedighemer and Laughon, 1993); stat92E is a hypomorph (Baksa et al., 2002). Rabsx-5\( ^{ex42} \) is a hypomorph (a gift from Cathie Pfeiffer, Yan et al., 2010). Transgenes used were UAS-Rab5x-5; Myc and UAS-Rab5x-5\( ^{DPP} \); Myc (gifts from Cathie Pfeiffer and Yan et al., 2010). UAS-YFP-Rab5\( ^{542N} \) (Bloomington and Zhang et al., 2007). UAS-Rbpn-5 was constructed by insertion of the Rbpn-5 coding region from the cDNA clone LD31555 (BDGP) into pUAST (Brand and Perrimon, 1993). pWIZ-Rbpn-5 is an RNAi line with no predicted off-targets and no homology to existing VDRC RNAi lines made by double inverted cloning of a 312 bp fragment of Rbpn-5 into the pWIZ vector (Lee and Carthew, 2003) using the following primers:

\[ \text{LeftRNAICG4030} + \text{Xbal:} \]

\[ \text{GCATCTAGACACACCTAATGGTGGCC} \]

\[ \text{RightRNAICG4030} + \text{Xbal:} \]

\[ \text{GCTCTAGAAGGTCGTTACGGGAG} \]

Transgens were generated by BestGene.

RNAi screen

An in vivo RNAi screen was carried out using approximately 10,000 lines from Vienna Drosophila Research Centres (VDRC) and National Institute of Genetics (NIG) (Helen Strutt, Vickie Thomas-MacArthur, CT and DS, unpublished data). Rabaptin-5 is a Rab5 effector protein that has been shown to physically interact with activated Rab5 and promote homotypic early endosome fusion in the mammalian tissue culture (Horiuchi et al., 1997; McBride et al., 1999; Stemmark et al., 1995; Vitale et al., 1998). The latter function of Rabaptin-5 is mediated through formation of a complex with the Rab5 guanine nucleotide exchange factor (GEF) Rabex-5, which acts to increase nucleotide exchange on Rab5, enhancing its activity and hence its ability to recruit other effectors involved in the endosomal fusion process (Horiuchi et al., 1997; Lippe et al., 2001). In addition to Rabex-5, a large number of other binding partners for Rabaptin-5 have been characterised to date, and it appears that it can act divalently to bridge different trafficking pathways. For example, through interacting with both Rab5 and Rab4 it mediates the transfer of cargo from early endosomes back to the plasma membrane through the fast recycling pathway (Daro et al., 1996; van der Sluijs et al., 1992; Vitale et al., 1998). Unlike most of the endocytic tumour suppressor genes so far identified, Rabaptin-5 has been implicated in human cancers, (Christoforides et al., 2012; Magnusson et al., 2001; Wang et al., 2009; Xiao et al., 1997), and thus the Drosophila mutant may represent a valuable model for the study of human tumourigenesis.

We find that several functions of Rabaptin-5 are conserved in Drosophila, including its GTP-dependent binding of Rab5, its ability to modulate endosomal dynamics and its interaction with Rabex-5. Our work suggests that Drosophila Rabex-5 is also a neoplastic tumour suppressor gene and that the tumour phenotype of both mutants is associated with ectopic activation of JNK and JAK/STAT pathways.

Materials and methods

Drosophila stocks and genetics

The following Drosophila stocks were used: From Bloomington stock centre (described in FlyBase): MS1096-GAL4, 459.2-GAL4, ptc-GAL4, pmr-GAL4, UAS-Drk2, cnwsp, P(fy \( \Delta (2-3) \)), Ubx-FLP, FRT42D P[arm-lacZ], P[arm-lacZ] FRT80B. P[Epy2]CG4030EY\( ^{1320} \) element, using cn and bw flanking markers for directional selection of recombination events. A small deletion, named CG4030Del1, was generated encompassing CG4030, CG4038 and CG34396 (sequence location 2R:16,976,092...16,989,307) and was found to be lethal if homoygous or transheterozygous with the larger deficiency Df(2)F36.

In order to rescue the entire CG4030Del1 deletion, the 33 kb attB-Pacman\( ^{CH322-86L04} \) construct was obtained from BAC PAC Resources and injected by Genetivision into the P2\( ^{3L} \)l68A4 site via a \( \Phi C31 \) transposase-mediated transgenesis. Rescued flies were viable and wild-type. Recombineering was used to generate a mutant form of attB-Pacman\( ^{CH322-86L04} \) lacking the CG4030 protein coding region, using methods based on those
described by the Bellen lab (Venken et al., 2006 and protocols at www.pacmanfly.org/protocols.html). The following oligos were used to amplify the loxP cassette from PL452N-EGFP:

RightCG4030arm+ + LoxF: 

TCATGCTGCTCCTGTTAATACAACTAACTAATCTGTTAATCAATCAGTAGCCCTCCCCGAGGAGAC

LeftCG4030arm+ + LoxPF: 

TGGATATCTGAAATGTCAGGAACAGGAGAGCT

The PCR fragment was recombineered into attB-PlacmaNCH322-86L04 and the loxP cassette was then flipped out to create attB-PlacmaNCH322-86L04-ΔGC4030. The construct was verified by sequencing and injected by Genetivision into the P2(3L)68A4 site via the Fc31 transposase-mediated transgenesis. Transgenic flies were used to create the following genotype for analysis of the Rbpn-5 mutant phenotype: w; FRT42 CG4030De11; attB-PlacmaNCH322-86L04-ΔGC4030/Sm6a;TM6b

Antibodies and Immunohistochemistry

The following primary antibodies were used for immunolabeling: 1:50 rabbit anti-Rab5 (a gift from Marcos Gonzalez-Gaitan (Wucherpfennig et al., 2003), 1:4000 rabbit anti-EGFP (Molecular Probes), 1:4000 rabbit anti-β-Gal (Cappel), 1:1000 mouse anti-flamingo (DSHB) (Usui et al., 1999), 1:300 rabbit anti-frizzled (Bastock and Strutt, 2007), 1:1000 rat anti-Strabismus (Strutt and Strutt, 2009). Imaginal discs were taken using the ProgResC14 camera system from Jenoptik on a Leica DMR upright microscope. Images were processed in ImageJ and figures constructed using Adobe Photoshop.

For Rab5 puncta analysis, a Z stack of 0.15 μm slices at 60× magnification with 3× zoom (0.069 μm per pixel) was obtained and slices were maximally projected in ImageJ as follows. Apical was defined as the three most apical in focus slices (roughly 0.5 μm thickness), whereas subapical was defined as the next 0.5 μm (slices 4–6). To get a value for total Rab5 (Fig. 4G), the top 6 slices were projected. Projected images were thresholded in ImageJ using the Triangle function and particles were analysed for their number, average size and total area in identically sized wild-type and mutant areas of the wing. T-tests were used to calculate statistical significance. Mutant values were normalised against wild-type values within each wing to make graphs. Data was compiled and statistically analysed using the Microsoft Excel.

Results

A screen for genes involved in planar polarity identifies the drosophila homologue of Rabaptin-5

We performed an in vivo RNAi screen in the Drosophila wing to look for novel genes involved in planar polarity (Helen Strutt, Vickie Thomas-MacArthur, CT and DS, unpublished data). An RNAi line targeting CG4030 showed a phenotype of mild wing trichome swelling, together with mild veining defects (VDRC line GD26367, Fig. S1B). CG4030 is predicted to encode a Rab5 binding protein with homology to Rabaptin-5 (Fig. 1A). Blast alignment indicates that CG4030 is likely to be the Drosophila Rabaptin-5 homologue (abbreviated here to Rbpn-5); protein homology is particularly high in the C-terminal region, which contains the putative Rab5 binding domain (NCBI Blast alignment score of 91.7), and is less conserved in the N-terminal region (NCBI Blast alignment score of 39.7). Unlike vertebrate Rabaptin-5 proteins, Drosophila Rbpn-5 has a FYVE (Fab1,YOTB/ZK63212,Vac1 and EEA1) domain at its extreme C-terminus. FYVE domains target proteins to endosomal membranes through binding to phosphatidylinositol 3-phosphate (PI3P), MgCl₂, and protease inhibitors. Beads were then incubated with GTP or GDP if required, for 90 min at room temperature in loading buffer containing 20 mM Hepes, 100 mM NaCl, 0.5 mM MgCl₂, 2 mM EDTA, 0.05% CHAPS, 1 mM DTT and protease inhibitors. 

EGFP-Rbpn-5 was cloned into the pAc5.1 vector (Invitrogen). The Rbpn-5 fragments Rbpn-5N (amino acids 1–345) and Rbpn-5C (amino acids 340–642) were made by removal of the unwanted half of the gene from pAc5.1-EGFP-Rbpn5 by restriction digestion, making use of the unique Xhol site halfway through the Rbpn-5 coding region. For Rbpn-5N a linker was added containing a STOP codon. Gene fragments were EGFP-tagged and inserted into pAc5.1. Vectors were transfected into Drosophila S2 cells. Cell lysate was extracted 48 h after transfection and added to the freshly prepared Gluthathione Sepharose 4B beads. Beads were incubated for 2 h at 4 °C buffer, then washed in buffer containing 250 mM NaCl to remove non-specifically bound proteins. Beads were loaded directly onto 10% acrylamide gels after denaturing at 95 °C for 3 min and Western blots were carried out using 1:4000 rabbit anti-EGFP (Abcam) or 1:5000 rat anti-Rbpn-5. Secondary antibodies used were anti-rat and -rabbit HRP (Dako) and signal was detected using Supersignal West Dura (Thermo Scientific).

Microscopy, image presentation and statistical analysis of puncta data

Fluorescent images were collected on an Olympus FV1000 confocal. Bright-field adult wing and notum photographs were taken using the ProgResC14 camera system from Jenoptik on a Leica DMR upright microscope. Images were processed in ImageJ and figures constructed using Adobe Photoshop.

For Rab5 puncta analysis, a stack of 0.15 μm slices at 60× magnification with 3× zoom (0.069 μm per pixel) was obtained and slices were maximally projected in ImageJ as follows. Apical was defined as the three most apical in focus slices (roughly 0.5 μm thickness), whereas subapical was defined as the next 0.5 μm (slices 4–6). To get a value for total Rab5 (Fig. 4G), the top 6 slices were projected. Projected images were thresholded in ImageJ using the Triangle function and particles were analysed for their number, average size and total area in identically sized wild-type and mutant areas of the wing. T-tests were used to calculate statistical significance. Mutant values were normalised against wild-type values within each wing to make graphs. Data was compiled and statistically analysed using the Microsoft Excel.

GST pulldown assays

The coding regions of Drosophila Rab4, Rab5, Rab5Q88L, Rab5N4435, Rab7, Rab8, Rab11, Rab23 and Rabex5 were individually cloned into the pGEX-6P-1 vector. GST-Rab proteins were expressed in E.coli, extracted from pelleted cells and bound to Glutathione Sepharose 4B beads (GE Healthcare) for 30 min at 4 °C in a buffer containing 50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM Glutathione Sepharose 4B beads (GE Healthcare) for 30 min at 4 °C, incubated with 2 h at 4 °C buffer, then washed in buffer containing 250 mM NaCl to remove non-specifically bound proteins. Beads were loaded directly onto 10% acrylamide gels after denaturing at 95 °C for 3 min and Western blots were carried out using 1:4000 rabbit anti-EGFP (Abcam) or 1:5000 rat anti-Rbpn-5. Secondary antibodies used were anti-rat and -rabbit HRP (Dako) and signal was detected using Supersignal West Dura (Thermo Scientific).
a lipid that is enriched on endosomal membranes (Burd and Emr, 1998; Stenmark et al., 1996). The Rbpn-5 FYVE domain appears to be conserved in many invertebrates, indicating that it may have been lost at some point in the vertebrate lineage (data not shown). Interestingly, another Rab5-interacting, FYVE domain-containing protein, EEA1, is not found in arthropods, although it is present in other invertebrates such as nematodes. However, sequence analysis suggests that Drosophila Rbpn-5 is only indirectly related to mammalian EEA1 through its FYVE domain and hence they are not likely to be orthologous (Fig. S1G).

To confirm the RNAi phenotype and check that the planar polarity phenotype was not due to an off-target effect, we made a...
In addition to trichome polarity and spacing defects (Fig. S1E-F), we also analysed the effect of RNAi expression in the notum using the panner-GAL4 (pnr-GAL4) driver. GD26367 RNAi-Rbpn-5 expression disrupts the polarity of microchaetae and trichomes in the notum, whereas pWIZ RNAi-Rbpn-5 expression causes loss of microchaetae in addition to trichome polarity and spacing defects (Fig. S1E-F). These pleiotropic results suggest that Rbpn-5 acts in multiple processes.

Rbpn-5 is a neoplastic tumour suppressor gene

In order to analyse the function of Rbpn-5 in more detail, we removed gene activity entirely using a small deletion of the Rbpn-5 gene region combined with p[acman] mediated rescue of flaming genes (see Section Materials and methods). Rbpn-5 mutants have an extended larval stage (approximately doubled compared with wild-type at 25 °C) in which they grow significantly larger than wild-type larvae, before dying shortly after pupation (Fig. 1B). This ‘giant larvae’ phenotype is reminiscent of that seen in neoplastic tumour suppressor mutants, a subgroup of which correspond to mutations in genes encoding endocytic proteins including Rab5 itself and another Rab5 binding protein, Rbpn-5 (Lu and Bilder, 2005; Morrison et al., 2008).

To test whether Rbpn-5 might be a novel neoplastic tumour suppressor gene, we examined wing and eye imaginal discs from Rbpn-5 giant larvae. Discs are variable in size and many are no larger than wild-type discs, however, tissue structure is disrupted and the epithelium is highly overgrown and fused in on itself (Fig. 1C-E). In addition, cell size appears to be variable, and many cells are extremely enlarged compared to wild-type (Fig. 1F-G). Neoplastic tumours are characterised by their absence of differentiation, disrupted apico-basal polarity and their upregulation of Matrix metalloprotease 1 (Mmp1), a target of JNK signalling involved in extracellular matrix remodelling, which is a marker for tumour metastasis (Beaucher et al., 2007; Page-McCaw et al., 2003; Srivastava et al., 2007; Uhliriova and Bohmann, 2006). Staining of eye discs with the neuronal marker Elav indicated that ommatidial differentiation does occur to some extent, but is not properly spatially regulated within the disc and differentiated cells appear abnormal in size and shape (Fig. 1E). Strong upregulation of Mmp1 levels was also seen, particularly in non-differentiated regions of the disc, indicating that tumours are likely to possess metastatic ability (Fig. 1E).

We examined the localisation of a large number of markers for apico-basal polarity, planar polarity and adherens junctions (Figs. 1F-J, S2A-F and not shown), all of which show a largely wild-type localisation pattern despite the disruption to tissue organisation. Unlike as described for other neoplastic tumour suppressors (Lu and Bilder, 2005; Menut et al., 2007; Moberg et al., 2005; Morrison et al., 2008; Rodahl et al., 2009; Vaccari and Bilder, 2005), it appears that apico-basal polarity is conserved in Rbpn-5 mutants, as we saw clear separation of markers for apical (aPKC) and baso-lateral (Dlg) membrane domains (Fig. 1H-I). Loss of apico-basal polarity in other endocytic neoplastic tumour suppressor mutants has been proposed to occur through an expansion of the apical domain caused by a failure to endocytose the apical determinant Crb (Lu and Bilder, 2005; Moberg et al., 2005; reviewed in Vaccari and Bilder, 2009). To test whether localisation of Crb was affected in Rbpn-5 mutants, we stained discs for Crb protein (Fig. 1J). Surprisingly, we do not see any significant accumulation of Crb in mutant discs compared to wild-type discs, suggesting that tumour formation can occur even when apico-basal polarity is not significantly disrupted.

A feature of neoplastic tumour suppressor genes is that clones of mutant cells surrounded by wild-type tissue do not overgrow, and in fact often proliferate less than the surrounding tissue (Agrawal et al., 1995; Brumby and Richardson, 2003; Igaki et al., 2006, 2009; Lu and Bilder, 2005; Ohsawa et al., 2011; Uhliriova et al., 2005). We generated mitotic clones of Rbpn-5 in Drosophila pupal wings using the FLP/FRT system and Ubx-FLP. Rbpn-5 mutant clones are viable and behave in a very similar way to wild-type cells, neither obviously over- nor under-proliferating. Moreover, no defects were seen in these clones compared to wild-type tissue when stained with a series of markers (Fig. 1K-L and not shown). In summary, these results suggest that Rbpn-5 is a novel neoplastic tumour suppressor gene that displays both similarities to and differences from neoplastic tumour suppressor genes already characterised.

Drosophila Rbpn-5 binds to Rab5

Mammalian Rabaptin-5 is considered to be an effector of Rab5 as it binds to it in a GTP-dependent manner in order to mediate certain functions including Rab5 recruitment and early endosome fusion (Stenmark et al., 1995). To test whether this function might be conserved in Drosophila, we carried out GST pulldowns to look for binding between Rbpn-5 and various Drosophila Rab GTPases. We found that as in vertebrates, Drosophila Rbpn-5 binds to Rab5 in the presence of GTP, or to an activated form of Rab5, but does not bind to Rab5 in the presence of GDP or to a dominant-negative form of Rab5 (Fig. 2A-B). To test where in the Rbpn-5 protein Rab5 might bind, we split Rbpn-5 into N- and C-terminal fragments (see Materials and Methods) and found as expected that Rab5 binds to the C-terminal part of Rbpn-5 (containing the predicted Rab5-binding domain) and not to the N-terminal fragment (Fig. 2C). We did not observe binding of Rbpn-5 to any other Rab protein tested including Rab4, Rab7, Rab11, Rab8 or Rab23 (Fig. 2B and data not shown). The absence of binding to Rab4 is surprising, since mammalian Rabaptin-5 does bind to Rab4 in a GTP-dependent manner to provide a link between endocytosis and the fast recycling pathway (Daro et al., 1996; Deneka et al., 2003; Pagano et al., 2004; van der Sluijs et al., 1992; Vitale et al., 1998). The putative Rab4 binding domain is in the N-terminal half of Rabaptin-5 (Vitale et al., 1998), which appears to be less highly conserved in invertebrates. This result mirrors that seen with Rbsn-5, another Rab5 effector whose mammalian homologue binds to Rab4 in vertebrate cells, but whose Drosophila homologue does not (de Renzis et al., 2002; Morrison et al., 2008; Nielsen et al., 2000), suggesting that the fast recycling pathway may not be regulated in the same manner in Drosophila as it is in mammalian cells.

Drosophila Rbpn-5 modulates early endosome dynamics in vivo

We raised an antibody against Drosophila Rbpn-5 (see Materials and Methods) and observed its localisation in pupal wings. Rbpn-5 is uniformly expressed and localises to punctate apical structures together with some cytoplasmic staining deeper within the cell (Fig. 3A–C). The punctate staining co-localises well with an anti-Rab5 staining antibody (Figs. 3A–B and data not shown). The punctate staining co-localises well with an anti-Rab5 staining antibody (Figs. 3A–B and data not shown).
We also made constructs to over-express Rbpn-5 under control of the UAS promotor. In mammalian cells, it has been shown that transfection of Rabaptin-5 results in excessive homotypic early endosome fusion, resulting in a characteristic enlarged early endosome phenotype (Stenmark et al., 1995). We observed a much more subtle phenotype in the Drosophila wing, whereby the number and size of subapical early endosomes is slightly, but significantly increased (Fig. 3F,H). However, we did also observe a slight reduction in the numbers of apical early endosomes. These results taken together suggest that in Drosophila, Rbpn-5 is necessary to promote normal levels of early endosome fusion, but is not sufficient to enhance the process by more than a very small amount, possibly due to limiting quantities of other proteins involved in early endosome fusion.

If a lack of Rbpn-5 significantly influences early endosome dynamics, then we would expect to see defects in the localisation of proteins that are normally endocytosed through this pathway. To this end, we examined a large number of markers in pupal wings expressing RNAi-Rbpn-5 under the ptc-Gal4 driver and compared them to adjacent wild-type cells. However, we were unable to find significant differences in localisation of any protein examined including those involved in apico-basal polarity or planar polarity, either in early or late pupal wings (Fig. S3 and data not shown). This suggests that despite the reduction in apical Rab5-positive vesicles, a sufficient level of endocytosis for normal function is still present in RNAi-Rbpn-5 expressing tissue.

**Drosophila Rbpn-5 and Rabex-5 interact to modulate early endosome dynamics**

In vertebrates, Rabaptin-5 acts to recruit activated Rab5 to endocytic sites through binding to and enhancing the activity of Rab5, a GTP exchange factor (GEF) that converts inactive GDP bound Rab5 to the active GTP bound form (Esters et al., 2001; Horiuchi et al., 1997). To test whether this interaction might be conserved in Drosophila, we carried out GST pulldowns between Rbpn-5 and the Drosophila homologue of Rabex-5, and found that the two proteins interact physically (Fig. 4A). Mammalian Rabex-5 binds to a coiled-coil region in the third quarter of Rabaptin-5 (Mattera et al., 2006), which has homology to the central region of Rbpn-5 (Fig. 1A). Since this domain is split between the N- and C-terminal halves of Rbpn-5 that we previously generated, we did not anticipate that Rabex-5 would be able to pull down either half individually, and indeed we could not demonstrate binding to either fragment (data not shown).

In *vitro* assays have shown that Rabaptin-5 requires Rabex-5 both for its recruitment to early endosomes and its ability to promote early endosome fusion, whereas Rabex-5 alone can stimulate excessive early endosome fusion if expressed at high enough levels (Horiuchi et al., 1997; Lippe et al., 2001). We obtained RNAi and UAS lines to examine the effect of Rabex-5 on early endosome dynamics in the Drosophila wing (VDRC and Yan et al., 2010). As with RNAi-Rbpn-5, apical Rab5 puncta are significantly reduced in RNAi-Rabex-5 expressing tissue (Fig. 4B). We also observed reduced levels of Rab5 (Fig. 4B), suggesting that as in *vitro*, Rabex-5 is required to recruit Rab5 to apical endosomes in Drosophila. Conversely, over-expression of Rabex-5 causes a large increase in both size and number of apical and sub-apical early endosomes (Fig. 4C,G). Furthermore, co-expression of UAS-Rbpn-5 enhances this phenotype (Fig. 4D,G), indicating that the two proteins can act together synergistically to promote early endosome fusion, and suggesting that the inability of UAS-Rbpn-5 to have much effect alone may be due to limiting levels of Rabex-5 normally present in the Drosophila wing. To test the requirement for Rbpn-5 in Rabex-5-mediated early endosome fusion, we removed Rab5n-5 function using RNAi, whilst also expressing...
Fig. 3. Rbpn-5 modulates early endosome dynamics in the Drosophila wing. (A–C) Rbpn-5 colocalises with Rab5 in Drosophila early pupal wings. Apically, Rbpn-5 colocalises with Rab5 in puncta, suggesting that Rbpn-5 localises to early endosomes (A, zoom in C). Medially (B), Rbpn-5 and Rab5 are predominantly cytoplasmic. Rab5 levels are reduced with little punctate staining and little co-localisation is seen. (D) Expression of RNAi-Rbpn-5 almost eliminates Rbpn-5 staining and strongly reduces apical Rab5 puncta (top half of each image). Subapical Rab5 puncta are not strongly affected. (E) Rbpn-5 mutant Ubx–FLP generated clones (marked by an absence of β-Gal in blue) show a similar reduction in Rbpn-5 staining and loss of apical Rab5 puncta. (F) Over-expressing Rbpn-5 results in a slight increase in number and size of subapical Rab5 puncta (top half of each image). Apical Rab5 puncta are slightly reduced. In all images (A–F) distal is to the right and anterior is upwards. Rbpn-5 staining is marked in red and Rab5 staining in green. Apart from E, images show an area distal to the posterior crossvein, with the L3 longitudinal wing vein extending horizontally along the centre of the image. UAS and RNAi constructs are expressed with the ptc-Gal4 driver above L3 (top half of the image), whilst tissue below L3 is wild-type (lower half of the image). UAS-Dicer2 was co-expressed with RNAi constructs to enhance efficacy. Wings were dissected at 4.5 h APF at 29°C. Scale bars are 5 μm long. (G–H) Quantification of Rab5 vesicle number and size in RNAi-Rbpn-5 and UAS-Rbpn-5 relative to wild-type shows that apical puncta are significantly reduced in both number and size. (I) Quantification of UAS-Rbpn-5 expressing tissue relative to wild-type shows that subapical vesicles are significantly increased in both number and size, but that slightly fewer apical vesicles are also present.
ectopic Rabex-5. Enlargement of early endosomes is still seen to a similar level as with UAS-Rabex-5 expression alone (Fig. 4E,G), indicating that excess Rabex-5 has the ability to promote early endosome fusion even when Rbpn-5 is depleted. We also expressed a mutant version of Rabex-5 that lacks GEF activity, UAS-Rabex-5DPYT (Yan et al., 2010). As expected, no increase in
early endosome size or number is observed and in fact levels of apical Rab5 puncta are severely reduced (Fig. 4F), indicating that Rab5 recruitment and early endosome fusion are dependent upon the GEF activity of Rabex-5. These results are in agreement with previous work from tissue culture (Horiuchi et al., 1997; Lippe et al., 2001), and suggest that the Rbpn-5/Rabex-5 complex is likely to be physically and functionally conserved in Drosophila.

Rabex-5 is a neoplastic tumour suppressor gene

A Rabex-5 hypomorphic mutant has previously been characterised and shown to form giant larvae or pupae, which often contain melanotic tumours (Yan et al., 2010). Through several pieces of evidence, these phenotypes have been attributed to misregulation of Ras signalling, which is known to promote growth and cause melanotic tumours (Yan et al., 2010; Zettervall et al., 2004). Rabex-5 regulates Ras signalling not through its GEF activity, but through a separate ubiquitin ligase domain (Mattera et al., 2006; Xu et al., 2010; Yan et al., 2010), although since ubiquitinated cargo including Ras are then targeted to early endosomes, in practice the two functions of the protein are likely to be highly interdependent (Aikawa, 2012; Aikawa et al., 2012; Mattera and Bonifacino, 2008).

Although Ras signalling promotes hyperplastic growth rather than neoplastic growth, the similarity of the described Rabex-5 mutant phenotype to that of other endocytic tumour suppressor genes led us to investigate the mutant animals more closely. As previously described (Yan et al., 2010), we observed that Rabex-5 homozygous or Rabex-5Δ3L/Δ3L transheterozygous mutant larvae grow excessively due to delayed pupation, and contain occasional melanotic tumours (data not shown). We examined imaginal discs and found that as for Rbpn-5, the disc epithelium of Rabex-5 mutants is overgrown and folded, cell size is increased and tissue labels strongly for Mmp1 expression (Fig. 5A–B). Eye-antennal discs are often fused with the brain lobes, Elav staining is variable and sometimes completely absent (e.g. Fig. 5A), and cell division is no longer clearly regulated (Fig. S4A), indicating that differentiation is compromised. However, again apico-basal polarity appears to be maintained in Rabex-5 mutants, and cell polarity markers such as aPKC are normal (Fig. 5A–C).

Fig. 5. Rabex-5 is a neoplastic tumour suppressor gene. (A) Rabex-5Δ3L/Δ3L, ptc-Gal4 eye discs stained for aPKC, Mmp1 and Elav. Differentiation is absent and Mmp1 levels are highly upregulated. Anterior is to the left. Scale bar is 50 μm long. (B–C) Wing discs stained with aPKC in red (apical), E-Cad in blue (junctional) and Dlg in green (baso-lateral) in Rabex-5 mutants. As seen in Rbpn-5, cells have a larger apical surface (B) but apico-basal polarity markers localise correctly at cell edges and show clear separation in a lateral view (C). Scale bars are 5 μm long. (D) Wild-type (D) and Rabex-5 mutant (D’) wing discs stained for Crb. Laser power and gain was kept at the same level for both images. Scale bars are 5 μm long. (E) Localisation of markers is normal in Rabex-5 mutant clones visualised in wings at 28 h APF. E-Cad is green and Fmi is red. Clones are marked by absence of β-Gal staining (blue). Scale bar is 10 μm long.

Fig. 4. Rbpn-5 binds to Rabex-5 and together they modulate early endosome dynamics in vivo. (A) GST pulldown showing binding of EGFP-Rbpn-5 to GST-Rabex-5 on a Gluthathione agarose column (see Section Materials and Methods for details). The blot was probed with anti-Rbpn5, which recognises EGFP-Rbpn-5 and (weakly) GST. (B) Expression of RNAi-Rabex-5 strongly reduces Rbpn-5 staining and apical Rab5 puncta (top half of each image). (C) Over-expressing Rabex-5 results in a strong increase in Rab5 positive vesicles, suggesting that Rabex-5 promotes early endosome fusion. (D) Co-expression of Rabex-5 and Rbpn-5 causes a synergistic increase in Rab5 recruitment to puncta. (E) Depleting Rbpn-5 activity using RNAi does not reduce the ability of excess Rabex-5 to recruit Rab5 to puncta. (F) Expression of a mutated Rabex-5 that lacks GEF activity causes a strong reduction in Rab5 puncta, indicating that Rab5 recruitment is dependent upon the GEF activity of Rabex-5. Images B–F are presented as described for Fig. 3 and scale bars are 10 μm long in all. The over-expression of Myc-tagged Rabex-5 constructs with ptc-Gal4 was verified by anti-Myc staining in blue. (G) Quantification of Rab5 puncta in the above genotypes. Quantification and statistical analysis was carried out as described in Section Materials and Methods. The value of puncta area is a factor of both puncta number and size. Mean values for puncta area were normalised to wild-type before plotting on a graph. Stars on bars indicate statistical difference between wild-type and mutants calculated from the raw data using paired t-tests, whereas stars above two bars represent statistical difference between two mutants, calculated from normalised data using an unpaired t-test. Three stars = p < 0.001.
occurs in a fairly wild-type fashion, but tissue is still abnormally folded (Fig. 5A–E). In wild-type wing discs, a ring of 10xSTAT-GFP is seen around the wing pouch, in the region corresponding to the wing hinge (D). Massive levels of 10xSTAT-GFP are observed in Rabex-5 mutant wing discs (D′, E). In wild-type wing discs, activated JNK (pJNK) is present at fairly low levels throughout (E), whereas in Rabex-5 mutant wing discs, pJNK levels are increased (E′). (F) A hypomorphic mutation in stat92E rescues various aspects of the neoplastic tumour phenotype as demonstrated by E-Cad staining. Unlike Rabex-5 single mutant eye discs (F), Rabex-5, stat92E double mutant eye discs do not invade the brain, are not overgrown or misshapen and ommatidial differentiation occurs in a fairly wild-type fashion, but tissue is still abnormally folded (F′).

since clear separation of apical, junctional and baso-lateral markers is seen and Crb levels are not increased compared with wild-type (Fig. 5B–D). Ubx-FLP-induced clones lacking Rabex-5 function were also generated. Cells within clones do not over-proliferate compared to twin-spots, and tissue appears wild-type with respect to a range of markers analysed (Fig. 5E and not shown). Together, these results indicate that Rabex-5 is a neoplastic tumour suppressor gene.

**Drosophila** neoplastic tumour phenotypes have been attributed to several mutually non-exclusive mechanisms. Mis-regulation of apico-basal polarity due to apical accumulation of Crb protein has been proposed as key step in tumourigenesis in early endocytic mutants (Lu and Bilder, 2005; Moberg et al., 2005; Vaccari and Bilder, 2009). Excess Crb is able to activate the Hippo (Hpo)/Warts (Wts) signalling pathway, thus promoting growth (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010; Robinson and Moberg, 2011). However, neither Rbpn-5 nor Rabex-5 mutant discs exhibit apico-basal polarity defects, and Crb does not significantly accumulate (Figs. 1F–J, 5B–D), indicating that this mechanism is not obligatory for neoplastic tumour formation. Hpo/Wts signalling is active throughout mutant discs as shown by an expanded-lacZ (ex-lacZ) reporter (Fig. S4B, Boedigheimer and Laughon, 1993), suggesting that this pathway might be contributing to disc growth. However, the effects are relatively mild and it is difficult to ascertain whether the broader expression domain compared to wild-type discs is significant or simply a side-effect of impaired differentiation.

In addition to Crb, other molecules have been found to accumulate apically in endocytic mutants, and ectopic activity of many signalling pathways has been documented in neoplastic tumours, including N, JNK and JAK/STAT. In order to try to investigate the mechanism of the neoplastic tumour phenotypes in more detail, we decided to look at the expression of a range of markers for activation of different signalling pathways. N has been shown to accumulate apically in endocytic mutants and in subapical vesicles in ESCRT mutants, with only the latter resulting in N pathway activation (Herz et al., 2006, 2009; Lu and Bilder, 2005; Moberg et al., 2005; Morrison et al., 2008; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2008). ESCRT mutant clones display a non-autonomous phenotype whereby ectopic N signalling promotes expression of the JAK/STAT ligand Upd, which then promotes growth in adjacent wild-type cells (Herz et al., 2006; Moberg et al., 2005; Vaccari and Bilder, 2005). We do not observe N accumulation in either Rbpn-5 or Rabex-5 mutant discs (Figs. 6A–B, S2C), and unsurprisingly, no increase is seen in expression of the N reporter Gbe-Su(H)-lacZ (Furriols and Bray, 2001, Fig. 6C), indicating that the N pathway is not ectopically activated. However, we do observe massive upregulation of JAK/STAT pathway activity in both mutants as assayed using a 10xSTAT-GFP reporter line (Bach et al., 2007, Figs. 6D, S4C).

In order to test whether JAK/STAT signalling plays an instructive role in tumourigenesis, we used a hypomorphic stat92E mutation (Frankenstein–stat92Efr) to try to rescue the tumour phenotype of Rabex-5 mutant discs. stat92Efr homozygotes are...
viable as adults, but produce knobly outgrowths on the dorsal thorax (Baksa et al., 2002 and data not shown). We found that like Rabex-5 single mutants, Rabex-5, stat92E homozygotes die during pupation, but there is no over-growth of either larvae or imaginal discs (Fig. 6F and data not shown). The tumorous phenotype of double mutant discs is significantly milder than that of single mutants, as eye-antennal discs are no longer fused to the brain, but show a relatively wild-type shape and structure, together with clear signs of differentiation (Fig. 6F). However, abnormal folds are still observed suggesting that not all aspects of tumourigenesis are downstream consequences of ectopic JAK/STAT pathway activity.

As Rabex-5 and Rbpn-5 mutants both show upregulation of the JNK target gene Mmp1 (Figs. 1E, 5A), it appeared likely that ectopic activation of the JNK pathway could also be contributing to tumour formation. We tested this using an antibody against activated (phosphorylated) JNK (pJNK) and indeed found a patchy accumulation of protein in Rabex-5 and Rbpn-5 mutant discs (Figs. 6E, 5A-D). In addition, we observed a variable increase in expression of the JNK lac-Z reporter (Martin-Blanco et al., 1998, Fig. 5A), suggesting that there is a significant upregulation of the JNK pathway in mutant discs, although it does not appear to be as dramatic as ectopic activation of the JAK/STAT pathway.

In summary, these results suggest that the neoplastic tumour suppressor phenotypes are linked to activation of JNK and JAK/STAT pathways, but are not caused by defective N signalling, or disrupted apico-basal polarity.

Discussion

Rbpn-5 and Rabex-5 are novel tumour suppressor genes

Endocytosis is increasingly recognised as an important mechanism for the growth and metastasis of tumours, both in the clinical setting and in cancer models. The categorisation of endocytic neoplastic tumour suppressor genes in Drosophila began less than ten years ago with the identification of Rab5, avl, Vps25 and Tsg101 (1a and Bilder, 2005; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005) and has since expanded with the discovery of at least 14 others, in particular multiple components of the ESCRT complex (Herz et al., 2009; Menut et al., 2007; Morrison et al., 2008; Rodahl et al., 2009; Vaccari et al., 2009). Most mutations so far identified are zygotic lethal long before the third instar larval stage and so have been discovered through screens in which homozygous mutant clones were generated that spanned entire epithelial compartments in otherwise heterozygous animals. However, not all chromosomal arms have been screened and screens have not been saturating, hence it is likely that many others remain to be found.

In this study we identify two novel endocytic neoplastic tumour suppressor genes. Rbpn-5 has not been previously characterised in Drosophila, and our work is the first to show that not only does it act as a neoplastic tumour suppressor gene in Drosophila, but that its endocytic functions, initially investigated in mammalian cell culture and in vitro (Stenmark et al., 1995), are conserved in a multicellular organism during development. Unlike many of the Drosophila endocytic neoplastic tumour suppressor genes so far identified, Rbpn-5 has a human homologue that has been directly implicated in tumour growth and metastasis. Mammalian Rabaptin-5 controls integrin recycling during migration of invasive tumour cells, has been identified in patients suffering from myelomonocytic leukaemia, is involved in preventing hypoxia in primary kidney and breast tumours and has been shown to interact physically with the Tuberous sclerosis protein Tuberin (Christoforides et al., 2012; Magnusson et al., 2001; Wang et al., 2009; Xiao et al., 1997). Although not all of these functions may be conserved in Drosophila, our work provides a basis for further investigating the mechanisms of Rbpn-5 dependent tumourigenesis in a whole animal system.

Rabex-5 has been previously identified as a tumour suppressor gene in Drosophila (Yan et al., 2010), but its neoplastic characteristics were not described. Importantly, both Rbpn-5 and Rabex-5 mutants are homozygous viable until late larval stages, and thus may provide a more facile model than other early endocytic tumour suppressor genes for uncovering the mechanistic basis of neoplasia.

We had initially set out to identify new regulators of planar polarity through an RNAi screen. As core planar polarity proteins are known to undergo internalisation and recycling (Mottola et al., 2010; Shimada et al., 2006; Strutt and Strutt, 2008; Strutt et al., 2011), and Rbpn-5 and Rabex-5 are general endocytic regulators that are functional in epithelial tissues at the time of planar polarisation, it is highly likely that they are playing a role in trafficking of core proteins. Indeed, another Rab5 effector, Rbsn-5, which is also a neoplastic tumour suppressor protein, regulates Fmi localisation (Morrison et al., 2008; Mottola et al., 2010). Unlike Rbsn-5, cell-autonomous depletion of Rbpn-5 or Rabex-5 does not significantly alter planar polarity protein levels or localisation (Figs. 1L, 5E, S3 and data not shown), suggesting that some level of endocytosis can occur in the absence of these proteins. One possibility is that Rbpn-5 and Rabex-5 proteins might be highly stable compared with Rbsn-5. Evidence in support of this comes from zygotic mutants, which survive to early pupal stages for Rbpn-5 and Rabex-5 (Fig. 1B, Yan et al., 2010), presumably due to perduration of maternal protein, but die before the second larval instar in the Rbsn-5 background (Morrison et al., 2008). If this is the case, residual protein activity in clones or RNAi-treated tissue may allow a low level of general endocytosis to occur, which would be sufficient to correctly localise core planar polarity proteins. Another, non-mutually exclusive hypothesis is that a level of endocytosis can occur in the absence of either Rabex-5 or Rbpn-5. There are three other Rab5 GEF homologues in Drosophila (Smythe, unpublished data), and these may be able to step in if Rabex-5 is absent.

Whatever the reason may be, the lack of defects seen in clones or RNAi, combined with the severe pleiotropic effects on epithelial architecture observed in zygotic mutants (Figs. 1C,E, 5A, and S2), means that analysis of a specific role in planar polarity is extremely complex and we decided to focus our attentions on their tumour suppressor roles.

The Rab5 effector function of Rabaptin-5 is conserved in drosophila

In mammalian cells, it has been shown that Rabaptin-5 is an effector of Rab5 that promotes early endosome fusion through its interaction with Rabex-5 (Horiiuchi et al., 1997; Lipie et al., 2001; McBride et al., 1999; Stenmark et al., 1995; Vitale et al., 1998). We provide here the first characterisation of a Rabaptin-5 homologue in a multicellular organism, and show that many of its functions are conserved. Rab5 effectors are defined by several criteria including GTP-dependent binding to Rab5 and the ability to modulate a Rab5-dependent process in response to Rab5 activity. We show here that Drosophila Rbpn-5 fulfills the criteria of a Rab5 effector protein in the same way as its mammalian counterpart.

Firstly we have demonstrated that Rbpn-5 physically interacts specifically with GTP-bound Rab5, probably through its predicted C-terminal Rab5 binding domain. Secondly, that Rbpn-5 and Rab5 colocalise in Drosophila pupal wings. Thirdly, when we deplete Rbpn-5 using RNAi or in mutant clones, we see a strong reduction in apical Rab5-positive vesicles indicating that Rbpn-5 is required for Rab5 recruitment. Lastly, if we over-express Rbpn-5 we observe
subtle alterations in early endosome structure, which are indicative of a role for Rbpn-5 in promoting early endosome fusion. Although the Rab5 effector function of Rabaptin-5 is conserved in Drosophila, its Rab4 effector function may not be. Mammalian Rabaptin-5 has been shown to bind Rab4 through a separate N-terminal domain, and the divergent Rab5-Rab4 binding ability allows Rbpn-5 to act as a bridge between the endocytic and fast recycling pathways (Daro et al., 1996; van der Sluijs et al., 1992; Vitale et al., 1998). We were not able to recapitulate Rab4 binding, and indeed the N-terminal Rab4 binding site is not highly conserved in Drosophila Rbpn-5. A similar result was found for the Drosophila homologue of another Rab5-Rab4 divergent effector, Rabenosyn-5 (Morrison et al., 2008), suggesting that the mechanism of transfer of cargo from the endocytic to recycling routes might not be conserved across phyla.

The interaction between Rabaptin-5 and Rabex-5 is conserved in drosophila

A large number of proteins have been shown to bind Rabaptin-5, but one of the best characterised is Rabex-5. The endogenous GEF activity of Rabex-5 is fairly low, and unless large quantities are overexpressed, the formation of a Rabaptin-5/Rabex5 complex is needed to promote nucleotide exchange on Rab5 (Esters et al., 2008), suggesting that the mechanism of transfer of cargo from the endocytic to recycling routes might not be conserved across phyla. Here we provide evidence that the interaction between Rabex-5 and Rbpn-5, and their ability to alter early endosome dynamics is conserved in Drosophila. As with their mammalian counterparts, the two proteins bind in vitro: we suspect that Rabex-5 binds to a central domain in Rbpn-5, as this is homologous to the Rabex-5 binding site in mammalian Rabaptin-5 (Mattera et al., 2006), and neither N- nor C-termini of Rbpn-5 can bind alone. As in mammalian cells (Horiiuchi et al., 1997; Zhu et al., 2007), overexpression of Rabex-5 in Drosophila wings is sufficient to promote early endosome fusion even if Rab5 levels are depleted by RNAi. However, co-expression of Rbpn-5 significantly enhances this, demonstrating that the complex acts synergistically. The dependency on Rabex-5 for Rabaptin-5 localisation to early endosomes is also conserved in Drosophila as we show that depletion of Rabex-5 levels using RNAi causes a reduction in Rbpn-5 puncta. This is surprising as not only does Rbpn-5 contain a Rab5 binding domain, but also a FYVE domain (unlike its mammalian homologue), which would be expected to promote its recruitment to early endosomes. It is possible that the FYVE domain is non-functional. Alternatively, its ability to bind PI3P may be somehow inhibited in the absence of Rabex-5, or the protein may be destabilised. Further work would be required to distinguish between these possibilities.

We found that over-expressing a mutant form of Rabex-5 lacking GEF activity (Yan et al., 2010) prevents both Rbpn-5 and Rab5 from accumulating in apical puncta. We surmise that the construct is acting in a dominant-negative manner to suppress Rbpn-5 recruitment and/or Rab5 activation via endogenous Rabex-5. In mammalian cells, localisation of all three proteins appears to be highly interdependent, suggesting that it is likely that Rabex-5 recruitment to early endosomes is also compromised in the absence of Rbpn-5 or Rab5. However, in the absence of reagents for visualising endogenous Rabex-5, this remains speculative.

Rbpn-5 and Rabex-5 neoplastic discs do not show disrupted apico-basal polarity

The mechanistic basis of neoplastic tumour formation is complex and much remains unclear. Drosophila models, in particular the classic baso-lateral polarity complex mutants scrib, dlg and lgl, which were discovered many years ago and which, unlike most of the endocytic mutants, are zygotically viable until late larval stages, have been extremely useful in describing the process of tumorigenesis (Bilder et al., 2000; Bilder and Perrimon, 2000; Bryant and Schubiger, 1971; Gateff, 1978; Gateff and Schneiderman, 1967; reviewed in Enomoto and Igaki, 2011). There are several different pathways and mechanisms that have been investigated. Given that Scrib, Dlg and Lgl regulate apico-basal polarity by inhibiting the apical Crb and Par-3/Bazooka (Baz) complexes (Betschinger et al., 2003; Bilder et al., 2003; Tanentzapf and Teapass, 2003), an expansion of the apical domain was proposed as one possible mechanism for tumourigenesis. This hypothesis was reinforced by the finding that over-expressing either Crb or atypical Protein kinase C (aPKC) is sufficient to promote tumourous discs, and that in the endocytic mutants, Crb accumulates massively and apico-basal polarity is disrupted (Bilder and Perrimon, 2000; Leong et al., 2009; Lu and Bilder, 2005; Moberg et al., 2005; Vaccari and Bilder, 2005). Crb accumulation is thought to promote the growth aspect of the neoplastic tumour phenotype through misregulation of the Hpo/Wts pathway (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Robinson et al., 2010; Robinson and Moberg, 2011). However, there is some evidence that Crb is not always strongly misregulated in neoplastic tumours, and it has been speculated that in scrib, dlg and lgl mutants it may be the concurrent disruption of endocytosis, rather than the polarity defect, which is the primary cause of their neoplastic phenotypes (Leong et al., 2009; Robinson and Moberg, 2011).

Our results show that neither Rabex-5 nor Rbpn-5 exhibit disruption in apico-basal polarity and there is no accumulation of Crb (Figs. 1F-J, 5B-D). We suggest that these mutants are likely to represent a milder phenotype than the other endocytic mutants studied so far, possibly due to enhanced perdurance of maternal protein which allows the survival of zygotic mutants to early pupal stages. In any case, the lack of an apico-basal polarity defect in Rbpn-5 and Rabex-5 mutants indicates that loss of apico-basal polarity is not strictly required for neoplasia.

Mechanisms of neoplastic tumour formation in Rbpn-5 and Rabex-5 mutants

Other pathways that have been found to be activated in Drosophila neoplastic tumours include N, JAK/STAT and JNK. Due to the differential ability of N to signal in different endocytic compartments, the pathway is upregulated in ESCRT mutant tumours, but not in early endocytic mutants, despite their accumulation of N protein on the cell surface (Herz et al., 2006; Herz et al., 2009; Lu and Bilder, 2005; Moberg et al., 2005; Morrison et al., 2008; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2008). As with Crb, we do not observe accumulation of N in Rbpn-5 or Rabex-5 mutant discs (Figs. 6A-B, and S2C), suggesting that a certain level of endocytosis is still occurring, and unsurprisingly we also find that the N pathway is not activated. In ESCRT mutant clones, activation of the N pathway leads to transcription of the JAK/STAT ligand Upd, which acts non-autonomously on wild-type tissue to induce JAK/STAT signalling and promote neoplasia (Herz et al., 2006; Moberg et al., 2005; Vaccari and Bilder, 2005). The massive upregulation of JAK/STAT signalling that we observe must be activated via an alternate mechanism, as it is both cell autonomous and N-independent. Although autonomous JAK/STAT signalling in neoplastic discs has been documented and shown to contribute to excessive cell size and proliferation (Gilbert et al., 2009; Woodfield et al., 2013), it is unclear how it is activated in endocytic mutants. One explanation that we were unable to test, is that the endocytic block could...
directly disrupt the trafficking of the JAK/STAT receptor Domeless (Dome), as Dome localisation has been shown to be altered in ESCRT mutant neoplastic tumourous discs (Gilbert et al., 2009), and signalling ability is known to be influenced by the intracellular compartment in which the ligand-receptor complex finds itself (Devergne et al., 2007; Vidal et al., 2010). We show that a hypomorphic stat92E mutation is able to rescue many of the neoplastic defects, including over-proliferation, inability to differentiate and overall disc shape and structure, suggesting that ectopic activation of JNK/STAT signalling in endocytic mutants is indeed causative for these aspects of tumourigenesis.

The upregulation of the JNK pathway that we observe in the Rbpn-5 and Rabex-5 mutants is likely to also contribute to the over-proliferation phenotype. The JNK pathway is unusual in having both pro-proliferation and pro-apoptotic roles, and was initially thought to be activated only where wild-type tissue abuts clones mutant for neoplastic tumour suppressor genes, promoting apoptosis within mutant cells and their elimination from the tissue (Brumby and Richardson, 2003; Igaki et al., 2006, 2009; Ohsawa et al., 2011; Uhlirrova et al., 2005). However, more recent work has shown that JNK signalling is also activated in tissues wholly mutant for endocytic neoplastic tumour suppressor genes where it promotes cell proliferation (Woodfield et al., 2013).

It is thought that JNK pathway upregulation in neoplastic tumours is likely to act through the Eiger/TNF ligand receptor complex (Igaki et al., 2002, 2009; Moreno et al., 2002). Eiger and activated JNK have been shown to accumulate in early endosomes in scribble mutant clones to promote apoptosis, and there are several other pieces of evidence that implicate the endocytic pathway in Eiger regulation (Igaki et al., 2009). Interestingly, the switch from JNK-mediated apoptosis to proliferation can be mediated by co-expression of the Ras oncogene. For example, expressing Ras in scribble mutant clones produces a highly invasive neoplastic phenotype such that clones that would usually be eliminated instead invade neighbouring wild-type tissue (Brumby and Richardson, 2003; Doggett et al., 2011; Igaki et al., 2006; Pagliarini and Xu, 2003; Uhlirrova et al., 2005; Wu et al., 2010). Given that Ras is ubiquitinated by Rabex-5, and the Ras pathway has been shown to be upregulated in Rbpn-5 mutant larvae (Xu et al., 2010; Yan et al., 2010), it is intriguing to speculate whether this may be contributing in some way to the neoplastic phenotype seen in mutant larval discs, not only in the Rabex-5 background but perhaps more generally in other endocytic neoplastic tumour suppressor mutants. Indeed, as Rabex-5-mediated ubiquitination targets cargo to early endosomes for degradation (Aikawa, 2012; Aikawa et al., 2012; Mattera and Bonifacino, 2008; Mattera et al., 2006), it seems highly likely that Ras signalling is not only regulated by ubiquitination but also by endosomal dynamics. Further work will be needed to investigate a potential role for Ras in endocytic neoplastic tumours, to determine the mechanisms of JNK and JAK/STAT activation and to elucidate how differential activity of these pathways is controlled in clones versus whole mutant tissues.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.09.029.

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