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RESEARCH ARTICLE

Drosophila Vps4 promotes Epidermal growth factor receptor signaling independently of its role in receptor degradation

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ABSTRACT

Endocytic trafficking of signaling receptors is an important mechanism for limiting signal duration. Components of the Endosomal Sorting Complexes Required for Transport (ESCRT), which target ubiquitylated receptors to intra-lumenal vesicles (ILVs) of multivesicular bodies, are thought to terminate signaling by the epidermal growth factor receptor (EGFR) and direct it for lysosomal degradation. In a genetic screen for mutations that affect *Drosophila* eye development, we identified an allele of *Vacuolar protein sorting 4* (*Vps4*), which encodes an AAA ATPase that interacts with the ESCRT-III complex to drive the final step of ILV formation. Photoreceptors are largely absent from *Vps4* mutant clones in the eye disc, and even when cell death is genetically prevented, the mutant R8 photoreceptors that develop fail to recruit surrounding cells to differentiate as R1-R7 photoreceptors. This recruitment requires EGFR signaling, suggesting that loss of *Vps4* disrupts the EGFR pathway. In imaginal disc cells mutant for *Vps4*, EGFR and other receptors accumulate in endosomes and EGFR target genes are not expressed; epistasis experiments place the function of *Vps4* at the level of the receptor. Surprisingly, Vps4 is required for EGFR signaling even in the absence of Shibire, the Dynamin that internalizes EGFR from the plasma membrane. In ovarian follicle cells, in contrast, *Vps4* does not affect EGFR signaling, although it is still essential for receptor degradation. Taken together, these findings indicate that Vps4 can promote EGFR activity through an endocytosis-independent mechanism.

KEY WORDS: Vps4, Endocytosis, EGF receptor, Dynamin, Signaling

INTRODUCTION

Endocytosis plays a dual role in signaling by many receptors; it is the route that leads to receptor degradation, but it can also alter the level of signaling activity by controlling receptor or ligand processing, recycling, localization or interaction with downstream components (Andersson, 2012; Callejo et al., 2011; Musse et al., 2012; Shilo and Schejter, 2011; Ueno et al., 2011). Activation of many receptors induces their ubiquitylation, internalization into early endosomes, sorting to multivesicular bodies (MVBs), where they are segregated from the cytoplasm, and ultimately lysosomal degradation (Piper and Lehner, 2011). The GTPase Dynamin catalyzes fission of Clathrin-coated endocytic vesicles from the plasma membrane (Schmid and Frolov, 2011). The subsequent sorting process is mediated by the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery (Hanson and Cashikar, 2012). ESCRT-0, which consists of the Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) and Signal transduction adaptor molecule (Stam) subunits, binds to and clusters ubiquitylated receptors. ESCRT-0 is recruited to endosomal membranes through interactions between Hrs and phosphatidylinositol 3-phosphate. It then recruits the four-subunit ESCRT-I complex, which brings in ESCRT-II, the substrate for assembly of ESCRT-III. The ESCRT-III subunit Vacuolar protein-sorting-associated protein 32 (Vps32) polymerizes into filaments that interact with Vps24 and Vps2 to deform the endosome membrane and promote budding of cargo-containing intra-lumenal vesicles (ILVs) (Henne et al., 2012; Wollert and Hurley, 2010). Vps2 also recruits the ATPase associated with a variety of cellular activities (AAA) protein Vps4, the only energy-utilizing ESCRT component (Hanson and Cashikar, 2012; Raiborg and Stenmark, 2009). Active Vps4 forms a hexameric complex that disassembles ESCRT-III, allowing recycling of its components, and also plays an active role in scission of the vesicle neck (Adell et al., 2014; Cashikar et al., 2014; Lata et al., 2008; Monroe et al., 2014; Mueller et al., 2012). In addition to their endocytic functions, ESCRT proteins, including Vps4, are required for cytokinesis, viral budding, protecting viral genomes from degradation, exosome secretion, receptor shedding on microvesicles, assembly of nuclear pore complexes, cholesterol transport and plasma membrane wound repair (Barajas et al., 2014; Choudhuri et al., 2014; Du et al., 2013; de Gassart et al., 2004; Jimenez et al., 2014; Morita, 2012; Nabhan et al., 2012; Tang, 2012; Webster et al., 2014).

The effect of endocytosis on signaling by the epidermal growth factor receptor (EGFR) is complex. Blocking EGFR internalization by removing Dynamin prevents its degradation, enhancing some downstream signaling events, but other aspects of EGFR signal transduction require an endosomal localization for the receptor (Jones and Rappoport, 2014; Legent et al., 2012; Miura et al., 2008; Teis et al., 2006; Vieira et al., 1996). Internalized EGFR can be either degraded or recycled to the plasma membrane, a choice that depends on the concentration and nature of the ligand, as ligands that remain bound in acidic late endosomes promote more extensive receptor ubiquitylation (Eden et al., 2012; French et al., 1995; Roepstorff et al., 2009; Sigismund et al., 2005). Recycling can alter the distribution of the receptor on the plasma membrane, controlling its exposure to ligands (Assaker et al., 2010; Jékely et al., 2005; Stetak et al., 2006; Vermeer et al., 2003). In *Drosophila*, EGFR targeted to the degradation pathway remains active on endosomes, as signaling is increased by ESCRT mutations that trap EGFR in the endocytic pathway (Vaccari et al., 2009). However, the transcriptional response in cultured mammalian cells depends primarily on EGFR activity at the plasma membrane (Brankatschk et al., 2012; Sousa et al., 2012). Observed effects on EGFR signaling could vary depending both on the stage at which endocytic sorting is blocked and the cellular context examined (Babst et al., 2000; Bache et al., 2006; Chanut-Delalande et al., 2010; Lloyd et al., 2002; Miura et al., 2008).

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In the *Drosophila* retina, EGFR signaling is essential for the differentiation of all photoreceptors except for R8, the first to differentiate in each ommatidium (Freeman, 1996). Adult eye phenotypes thus provide a rapid screen for new regulators of the EGFR pathway (Legent et al., 2012; Miura et al., 2008; Roignant et al., 2006; Roignant and Treisman, 2010). Here, we report that a mutation in *Vps4* disrupts EGFR signal transduction as well as the transduction of other signaling pathways. In *Vps4* mutant cells in the eye disc, EGFR accumulates in endosomes but is unable to signal, resulting in the failure of non-R8 photoreceptors to differentiate. Interestingly, removing the Dynamin encoded by *shibire* does not restore EGFR signaling in the absence of *Vps4*, suggesting a new role for Vps4 upstream of EGFR internalization. *Vps4* mutant ovarian follicle cells accumulate endocytosed EGFR, but show normal expression of EGFR target genes, indicating that defects in EGFR signaling in imaginal discs are not a consequence of its sequestration in the endocytic pathway. Taken together, these results suggest that Vps4 promotes EGFR activation independently of its effects on endocytosis.

RESULTS

Vps4 is required for R8 survival and R1-R7 differentiation

In a mosaic screen of the X chromosome (Legent et al., 2012), we recovered a mutation that prevents photoreceptor differentiation. In eye imaginal discs, clones of *3B1* mutant cells showed a cell-autonomous lack of expression of the pan-neuronal marker Elav (Fig. 1A), normally expressed by differentiating photoreceptors (Robinow and White, 1991). Genetic mapping and sequencing revealed that *3B1* was a missense mutation in the *Vps4* gene. 3B1 transforms glutamate 209, which is adjacent to the first central pore motif of the AAA domain (Scott et al., 2005), into a lysine residue (Fig. 1B), a charge reversal that would probably disrupt protein folding. *3B1* failed to complement the lethality of $Vps4^{\Delta 7b}$, a small deficiency that covers *Vps4* and its flanking sequences (Rodahl et al., 2009a). Expression of an HA-tagged wild-type *Vps4* cDNA in *3B1* clones fully rescued photoreceptor differentiation (Fig. 1C-E). Additionally, 98% (*n*=106) of hemizygous *3B1* males were rescued to viability by *tubulin-GAL4*-driven ubiquitous expression of UAS-*HA::Vps4*. These results confirm that the phenotypes observed in *3B1* mutants are due to loss of *Vps4* function.

The absence of photoreceptors in *Vps4* mutant clones could be due to either failure of differentiation or cell death. We observed that $Vps4^{3B1}$ mutant clones in third-instar eye discs underwent massive apoptosis, as indicated by their pyknotic nuclei and high levels of activated effector caspases (Fig. 2A). Both features were largely rescued in the absence of the initiator caspase *Dronc* (Steller, 2008) (Fig. 2B). Signaling pathways converging on c-Jun N-terminal Kinase (JNK) have been implicated in the regulation of programmed cell death in various contexts (Dhanasekaran and Reddy, 2008). As previously reported for *Vps4* RNAi expression (Rodahl et al., 2009a), we found that $Vps4^{3B1}$ clones misexpressed the JNK transcriptional target *puckered* (*puc*)-*lacZ* (Martin-Blanco et al., 1998) (supplementary material Fig. S1A). To assess whether ectopic JNK signaling is instructive in photoreceptor cell death, we created clones mutant for both *Vps4* and *hemipterous* (*hep*), which encodes the upstream activating kinase for JNK (Glise et al., 1995). However, removing *hep* did not restore photoreceptor differentiation or prevent caspase activation (supplementary material Fig. S1B-F). Similarly, inhibiting JNK signaling by overexpressing the JNK phosphatase Puc failed to rescue the *Vps4* phenotypes (supplementary material Fig. S1G,H). JNK activity is thus not the primary driver of apoptosis in *Vps4* mutant cells in the eye disc.

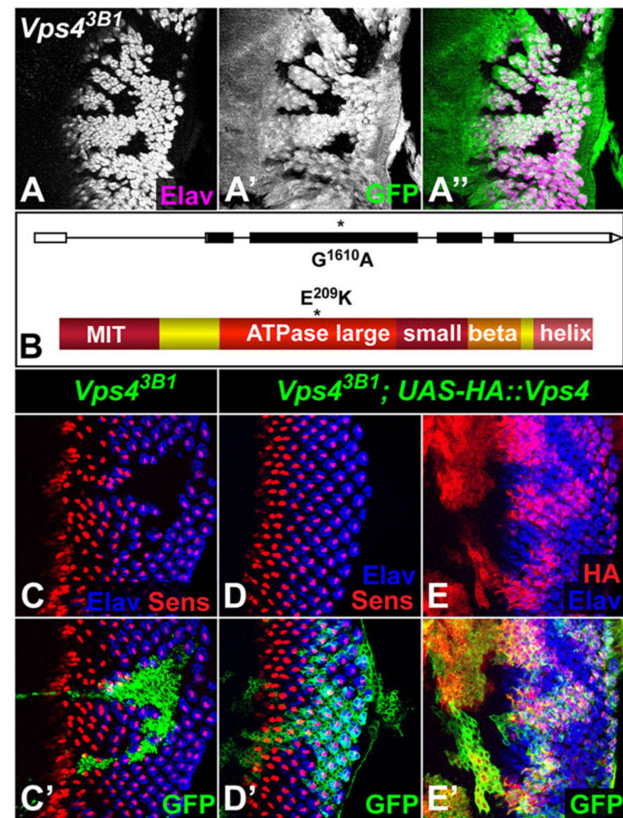

Fig. 1. *Vps4* is required for photoreceptor differentiation. (A) $Vps4^{3B1}$ mutant clones in third-instar eye discs, marked by the absence of GFP (A′, green in A″), lack photoreceptors stained with anti-Elav (A, magenta in A″). Anterior is to the left in this and all subsequent figures. (B) Diagram of the *Vps4* gene and encoded protein indicating that the 3B1 mutation changes glutamic acid 209 in the large AAA ATPase domain into a lysine residue. MIT, microtubule interacting and trafficking domain. The ATPase domain is subdivided into large, small and beta domains. (C-E) Eye discs expressing GFP (green) alone (C) or together with HA-tagged wild-type Vps4 (D,E), within $Vps4^{3B1}$ mutant clones. HA::Vps4 (stained for HA, red in E) rescues the differentiation of photoreceptors labeled with Elav (blue) including R8, which is marked by Sens (red in C,D).

The rescue of cell death observed in the absence of *Dronc* allowed us to examine early markers of photoreceptor differentiation in *Vps4* mutant cells. In each ommatidium, R8, marked by expression of Senseless (Sens) (Frankfort et al., 2001), is the first photoreceptor to differentiate. R8 cells are singled out of an anterior stripe of proneural precursors expressing Atonal (Ato) (Jarman et al., 1993). Only a few Sens-positive R8 cells were observed in anterior regions of $Vps4^{3B1}$ mutant clones (Fig. 2C). However, preventing cell death by removing *Dronc* rescued many R8 photoreceptors (Fig. 2D), indicating that *Vps4* is required for R8 survival. R8 induces EGFR activation in surrounding cells to promote their differentiation into R1-R7 photoreceptors (Freeman, 1997; Tio et al., 1994). In *Vps4* clones, most rescued R8 cells failed to recruit any Elav-positive neighboring cells (Fig. 2E,F). The absence of R1-R7 photoreceptors is thus not a secondary consequence of cell death, but results from a failure to transduce signals from R8.

Vps4 mutant cells accumulate inactive signaling receptors

Mutations in *Tumor susceptibility gene 101* (*TSG101*) of the ESCRT-I complex and *Vps25* of the ESCRT-II complex cause accumulation of the receptor Notch (N), which drives excessive

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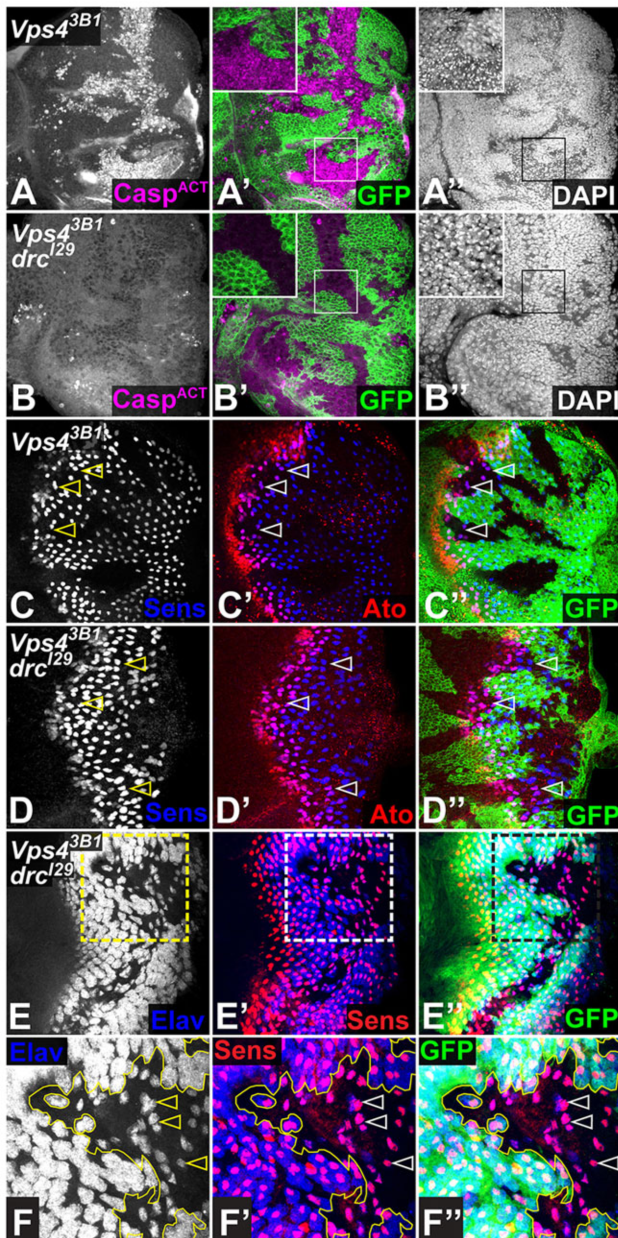

Fig. 2. *Vps4* is independently required for cell survival and R1-R7 differentiation. (A-F) Eye discs carrying $Vps4^{3B1}$ mutant clones marked by the absence of GFP (green), in a wild-type (A,C) or $Dronc^{l29}$ (drc^{l29}) (B,D-F) background. $Vps4^{3B1}$ clones display elevated levels of activated Caspase 3 (A, magenta in A′) and pyknotic nuclei stained with DAPI (A″), which are greatly reduced in $Dronc^{l29}$ homozygotes (B). Very few Sens-positive R8 cells (C, blue in C′,C″) differentiate in $Vps4^{3B1}$ clones (arrowheads). Expression of Ato (red) in anterior proneural precursors is decreased but not abolished. (D) A substantial rescue of differentiated R8 photoreceptors stained with Sens (blue) and Ato (red) is observed in $Dronc^{l29}$ homozygotes (arrowheads). (E,F) $Vps4^{3B1}$ clones in $Dronc^{l29}$ discs stained for Elav (E, blue in E′,E″) and Sens (red). (F) An enlargement of the region boxed in E. Within the $Vps4^{3B1}$ clone (outlined), R8 cells are not surrounded by Elav-positive photoreceptors (arrowheads).

signaling, leading to nonautonomous overproliferation (Moberg et al., 2005; Vaccari and Bilder, 2005). Similarly, we found that *Vps4* mutant cells accumulated very high levels of both N and its ligand Delta (Dl) in punctate structures (Fig. 3A,B). However, unlike the upstream ESCRT mutants *TSG101* and *Vps25*, N signaling was reduced in *Vps4* mutant cells, as visualized by lower levels of the transcriptional reporter *E(spl)mβ-CD2* (de Celis et al., 1998; Moberg et al., 2005) in the eye disc (Fig. 3C). We also investigated the requirement for *Vps4* in N signaling during wing development. As *Vps4* clones in the wing disc survived poorly (supplementary material Fig. S2), we induced them in a *Dronc* background, and again observed accumulation of N in large puncta (Fig. 3D). In the wing disc, N is activated in a stripe of cells along the dorsal-ventral (D-V) boundary, where it induces the transcription of *cut* (*ct*) (de Celis et al., 1996). Ct expression was lost in *Vps4* clones (Fig. 3E), confirming that, in contrast to more-upstream ESCRT mutants, *Vps4* promotes N signal transduction.

We next investigated whether other signaling pathways were similarly affected. In the wing disc, Wingless (Wg) secreted from both the D-V boundary and the hinge surrounding the wing pouch acts at a long range to organize wing growth and patterning (Neumann and Cohen, 1997). Like N and Dl, Wg accumulated in large punctate structures in *Vps4* mutant cells (Fig. 3F). In both the wing and eye discs, Wg only accumulated in mutant cells within or close to its endogenous domain of expression, which is marked by the reporter *wg-lacZ* (Fig. 3H-I). *wg-lacZ* was unaffected by the loss of *Vps4* (Fig. 3H), indicating that Wg protein accumulation results from internalization into receiving cells rather than increased transcription. *Vps4* clones showed reduced expression of the Wg target genes *sens* in the wing disc (Fig. 3G) (Parker et al., 2002) and *dachsous-lacZ* in the eye disc (Yang et al., 2002) (data not shown), demonstrating that normal Wg signaling requires *Vps4*.

In contrast to the reduction in N and Wg signaling, we observed increased levels of the second messenger phosphorylated Smad (pSmad) (Tanimoto et al., 2000) in *Vps4* mutant clones near the domain of expression of the Bone Morphogenetic Protein family member Decapentaplegic (Dpp) in the eye disc (Fig. 3J), suggesting that *Vps4* mutant cells are able to transduce some signals and that, in contrast to its effects on other pathways, Vps4 negatively regulates signaling by Dpp. Taken together, our results indicate that unlike more-upstream ESCRT members, Vps4 is required for the transduction of several signaling pathways.

Vps4 is required for EGFR signaling

The effect of receptor internalization and endocytic processing on EGFR signaling remains controversial. Although some studies have demonstrated that it contributes to signal termination through lysosomal EGFR degradation (Bache et al., 2006; Razi and Futter, 2006), other results argue that it enables receptor signaling from intracellular organelles (Miaczynska et al., 2004; Shilo and Schejter, 2011). *Vps4* clones in *Dronc* mutant eye discs resemble clones lacking positive intracellular effectors of EGFR transduction, in which R8 cells fail to recruit additional photoreceptors (Legent et al., 2012). We found that the expression of *lacZ* reporters for two direct transcriptional targets of the EGFR pathway in R1-R7, *argos* (*aos*) (Golembo et al., 1996) and *hedgehog* (*hh*) (Rogers et al., 2005), was downregulated in *Vps4* mutant clones (Fig. 4A,B). Importantly, restoring R8 survival by removing *Dronc* did not rescue *aos* expression in surrounding cells (Fig. 4C). In the wing disc, EGFR signaling controls both the expression of *aos* in vein primordia (Sturtevant et al., 1993), and the expression of *mirror* (*mirr*) in the notum primordium (Zecca and Struhl, 2002). Consistent with a requirement for Vps4 in EGFR signaling, *Vps4* mutant cells displayed a reduced expression of *aos-lacZ* and *mirr-lacZ* (Fig. 4E,F).

Despite the lack of EGFR target gene expression, EGFR protein levels were increased in *Vps4* mutant cells (Fig. 4D,G,H).

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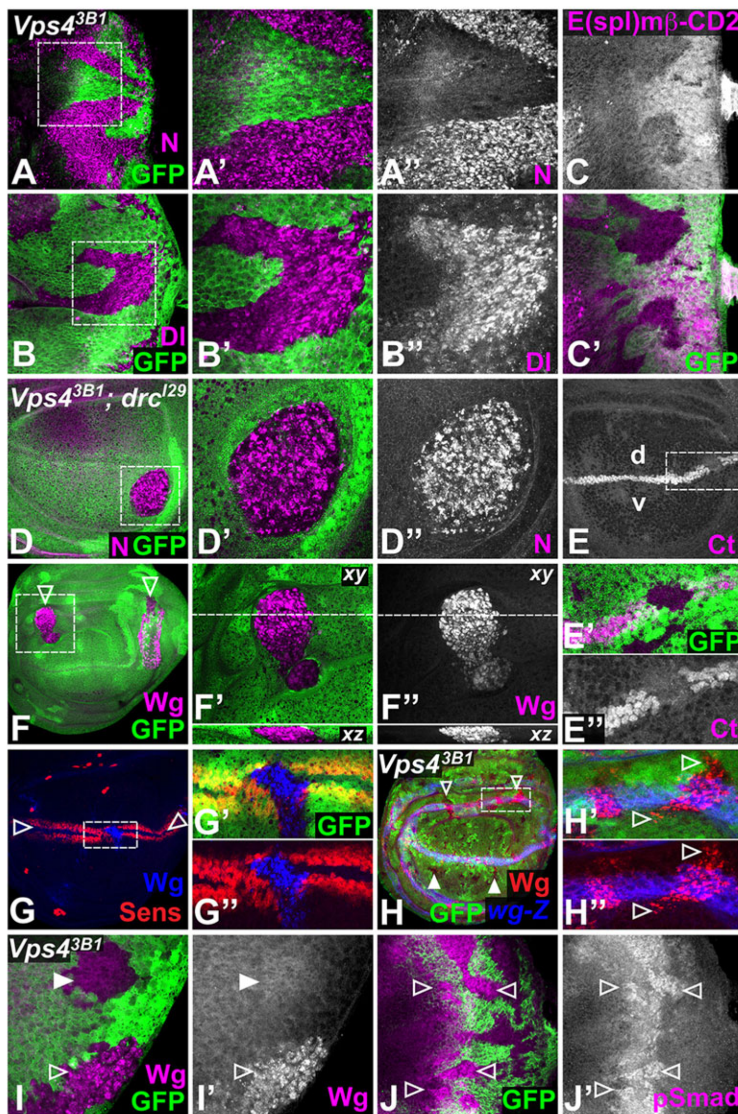

Fig. 3. Loss of *Vps4* affects multiple signaling pathways. *Vps4*^{3B1} clones marked by the absence of GFP (green) in eye discs (A-C,I-J) or in *Dronc*^{I29} (*drc*^{I29}) homozygous wing discs (D-H). (A,B) Mutant cells accumulate high levels of N (A″, magenta in A,A′) and Dl (B″, magenta in B,B′) in large puncta (boxed regions are enlarged in A′,A″,B′,B″). (C) Expression of the Notch transcriptional reporter *E(spl)-mβ-CD2* (C, magenta in C′) is decreased in *Vps4* clones. (D) Mutant cells accumulate large N-positive puncta (D″, magenta in D,D′, the boxed region is enlarged in D′,D″). (E) The Notch transcriptional target Ct (E,E″, magenta in E′), normally expressed at the boundary between the dorsal (d) and ventral (v) compartments of the wing pouch, is lost in *Vps4* clones (the boxed region is enlarged in E′,E″). (F) Wg (F″, magenta in F,F′) also accumulates in large puncta that span the depth of the cell in *Vps4* clones (arrowheads; the boxed region is enlarged in F′,F″; *xz* sections are shown below). (G) The Wg target Sens (red) is expressed in two stripes (arrowheads) along the D-V boundary of the wing pouch, but is lost from *Vps4* clones, despite accumulation of Wg (blue). The boxed region is enlarged in G′,G″. (H) Ectopic Wg (red) is observed in *Vps4* clones in the vicinity of Wg-producing cells (open arrowheads), marked by *wg-lacZ* (blue), but not in more distant clones (filled arrowheads). The boxed region is enlarged in H′,H″. (I) In eye discs, Wg puncta (I′, magenta in I) are observed in clones along the lateral margins (open arrowhead) but not in more medial clones (filled arrowhead). (J) *Vps4* clones display elevated levels of pSmad (J′, magenta in J) in the region of the morphogenetic furrow (arrowheads).

Intracellular EGFR colocalized with N in punctate structures, many of which also expressed the early endosomal markers Hrs, Rab5 and Syntaxin 7 (Syx7) (Lloyd et al., 2002; Lu and Bilder, 2005; Russell et al., 2012) (Fig. 4D,G,H; supplementary material Fig. S3A). Little colocalization was observed with markers for recycling endosomes (Rab11), the Golgi (Lava lamp; Lva) or secretory vesicles (Sec15) (Langevin et al., 2005; Sisson et al., 2000) (supplementary material Fig. S3B-D), arguing that the EGFR accumulation is in enlarged endosomes, known as class E compartments in yeast (Russell et al., 2012). Consistent with this view, loss of the ESCRT-I subunit TSG101 caused a similar accumulation of EGFR in punctate structures expressing Hrs (supplementary material Fig. S3F).

S2 cells stably expressing the EGFR (D2F cells) have been used to study EGFR signaling mechanisms (Schweitzer et al., 1995). Treatment of these cells with a purified secreted form of the EGFR ligand Spitz (Spi) (Miura et al., 2006) resulted in increased production and secretion of Aos protein (Fig. 4I). However, RNAi-mediated knockdown of *Vps4* (supplementary material Fig. S4A) strongly reduced this response (Fig. 4I). *Vps4* RNAi also reduced the phosphorylation of the downstream effector MAPK (also known as ERK and Rolled) in cells treated with Spi (supplementary material Fig. S4B). These results indicate that the effect of Vps4 on EGFR signaling is not dependent on the fate or epithelial organization of imaginal disc cells.

Vps4 acts at the level of EGFR activation

In order to determine at which step Vps4 influences EGFR signal transduction, we performed epistasis experiments. In the eye disc, Spi secreted by R8 is the primary ligand for EGFR in R1-R7 (Freeman, 1994; Tio et al., 1994). Its binding triggers receptor dimerization and activation by autophosphorylation, and subsequent recruitment of enzymes and adaptor proteins promotes conversion of Ras (also known as Ras85D) into its GTP-bound form. This small GTPase initiates a kinase cascade by activating Raf (also known as Pole hole), which phosphorylates MEK (also known as Dsor1), which in turn phosphorylates MAPK. Phosphorylated MAPK enters the nucleus and phosphorylates specific transcription factors to regulate target gene expression (Shilo, 2003). During eye development, expression of a secreted form of Spi (sSpi) (Schweitzer et al., 1995) or constitutively active forms of EGFR (Queenan et al., 1997) or Ras (Karim and Rubin, 1998) ectopically activates EGFR signaling and promotes the differentiation of extra photoreceptors (Fig. 5A-C). Expression of sSpi did not rescue photoreceptor differentiation in *Vps4* mutant cells, although it triggered ectopic photoreceptor

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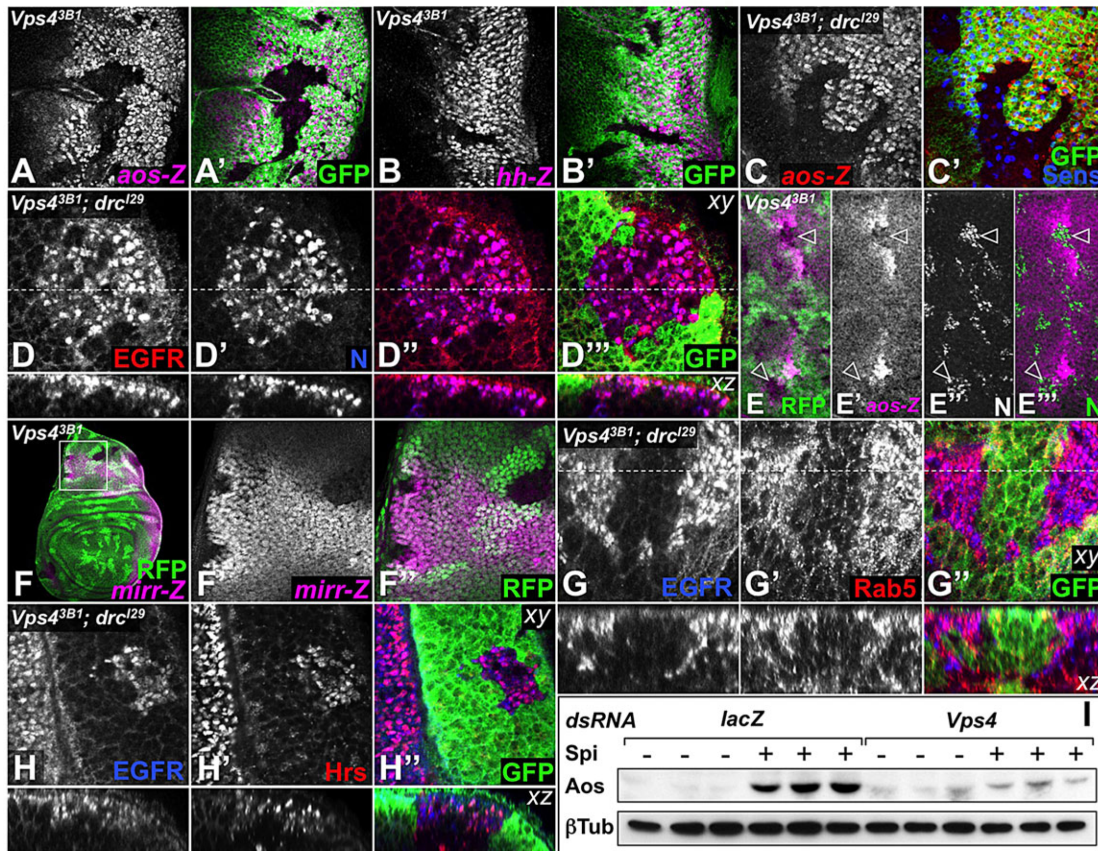

Fig. 4. *Vps4* is required for EGFR signaling. Eye discs (A-D,G-H) or wing discs (E,F) in which $Vps4^{3B1}$ mutant clones are marked by the absence of GFP (green, A-D,G-H) or RFP (green, E,F), in a wild-type (A-B,E-F) or $Dronc^{l29}$ (drc^{l29}) homozygous (C-D,G-H) background. $Vps4^{3B1}$ clones lose expression of the EGFR transcriptional targets *aos-lacZ* (A, magenta in A′) and *hh-lacZ* (B, magenta in B′). (C) R8 cells marked by Sens (blue) in *Vps4* clones in *Dronc* homozygotes fail to induce *aos-lacZ* (C, red in C′) in surrounding cells. (D) EGFR (D, red in D″,D‴) and N (D′, blue in D″,D‴) accumulate and colocalize in punctate structures in *Vps4* mutant cells (*xz* sections are shown below). (E) *aos-lacZ* (E′, magenta in E,E‴) is normally expressed along presumptive veins but is lost in $Vps4^{3B1}$ clones (arrowheads), which accumulate high levels of N (E″, green in E‴). (F) In the notum, expression of the EGFR transcriptional target *mirr-lacZ* (F′, magenta in F,F″) is decreased in *Vps4* mutant cells. The boxed region is enlarged in F′,F″. (G,H) In *Vps4* mutant cells, punctate accumulations of EGFR (G,H, blue in G″,H″) colocalize extensively with Hrs (H′, red in H″) and partially with Rab5 (G′, red in G″). Both *xy* and *xz* sections are shown. (I) Western blots of Aos in the culture media and β-tubulin in the cell lysates of triplicate samples of D2F cells treated with *lacZ* or *Vps4* dsRNA as indicated, and exposed (+) or not exposed (–) to purified sSpi for 16 h. *Vps4* knockdown strongly reduces Aos production.

differentiation in wild-type cells surrounding the clone, indicating that *Vps4* is not necessary for Spi secretion (Fig. 5D). The same result was obtained by overexpressing Rhomboid, a protease that activates endogenous Spi (Lee et al., 2001) (data not shown). Interestingly, a constitutively dimeric form of EGFR showed partial activity in *Vps4* mutant cells; although photoreceptors still failed to differentiate within *Vps4* mutant clones expressing activated EGFR, surrounding cells showed premature differentiation (Fig. 5E), probably due to *hh* expression induced within the clone (supplementary material Fig. S5). Expression of activated Ras was sufficient to bypass the requirement for *Vps4* in EGFR transduction and promote the differentiation of extra photoreceptors both within and surrounding the clone (Fig. 5F). Similar epistasis experiments in the wing disc, using *aos-lacZ* to monitor pathway activation, showed that activated EGFR promoted strong ectopic *aos* expression in wild-type but not *Vps4* mutant cells, whereas activated Ras could upregulate *aos-lacZ* even in the absence of *Vps4* (supplementary material Fig. S6). The activity of Vps4 is thus required in the receiving cell, upstream of Ras activation but downstream or at the level of EGFR activation (Fig. 5G).

Given that the EGFR pathway is required for cell survival in the eye disc (Halfar et al., 2001; Yang and Baker, 2003), we wondered whether restoring signaling downstream of the receptor could rescue the cell death observed in *Vps4* mutant clones. Indeed, although activated EGFR did not prevent caspase activation in *Vps4* cells (Fig. 5H-J), activated Ras abolished apoptosis (Fig. 5K,L). These results demonstrate that, rather than ectopic JNK signaling (supplementary material Fig. S1) (Rodahl et al., 2009a), impaired EGFR signal transduction is the primary reason for the death of *Vps4* mutant cells.

Vps4 functions upstream of EGFR endocytosis

Because the effect of Vps4 on EGFR signaling was opposite to the negative effect of other ESCRT components (Vaccari et al., 2009) (see supplementary material Fig. S3E), we wanted to test whether Vps4 acted during endocytosis to affect EGFR signaling. We therefore generated double mutant clones for *Vps4* and *shibire* (*shi*), which encodes Dynamin, a GTPase required to internalize EGFR and other receptors from the plasma membrane (Henriksen et al., 2013; Sousa et al., 2012; Windler and Bilder, 2010). shi^{7C7} mutant cells have enhanced EGFR signaling, leading to increased photoreceptor differentiation (Legent et al., 2012) (Fig. 6A). The ESCRT-0 component Hrs has a positive role in EGFR signaling and photoreceptor differentiation in the eye disc (Miura et al., 2008). As expected, given that Dynamin acts prior to Hrs in endocytosis, *shi*

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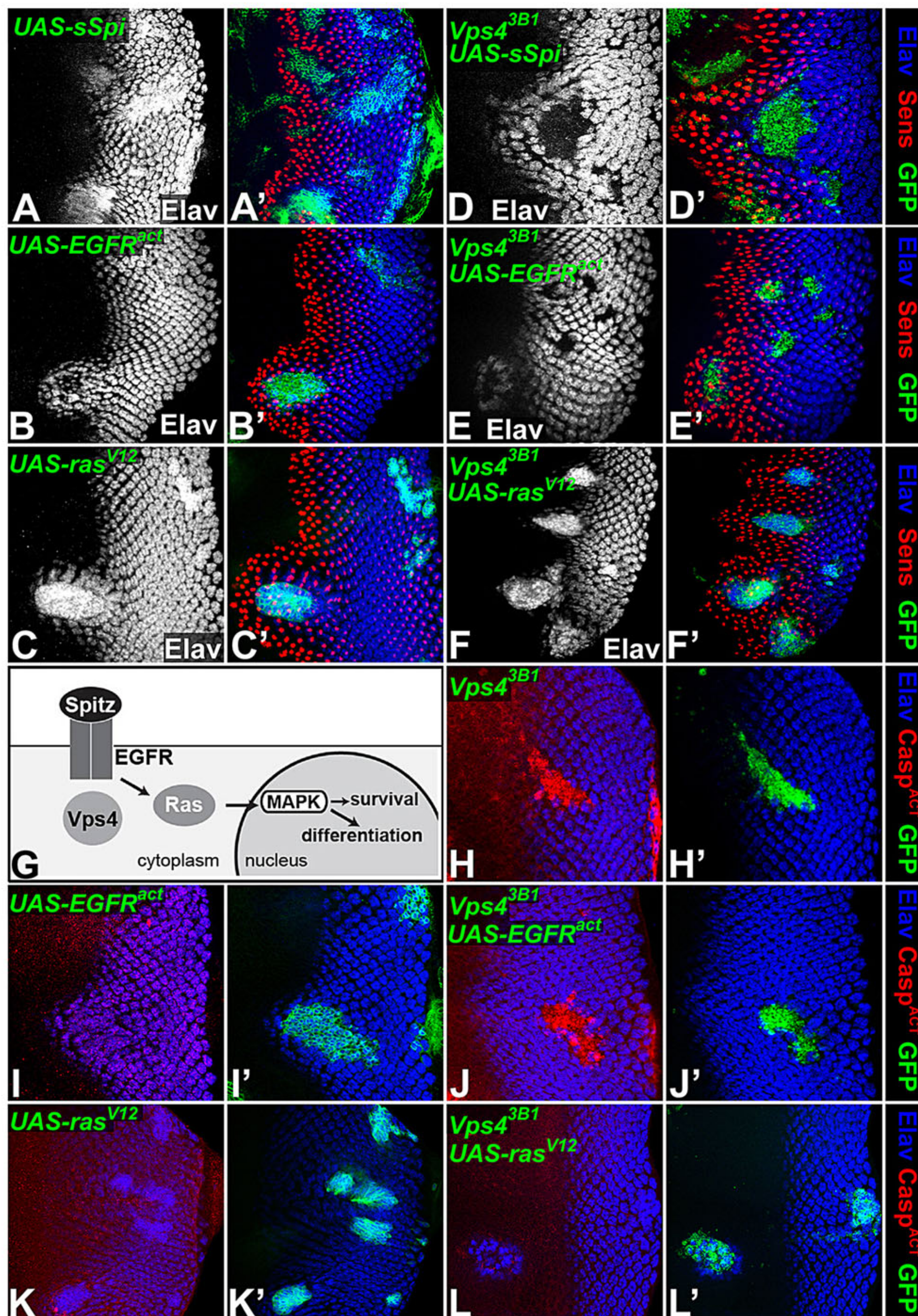

Fig. 5. *Vps4* acts at the level of the EGFR. (A-F,H-L) Eye discs with clones coexpressing GFP (green) with activated pathway components, stained for Elav (A-F and blue in A′-F′,H-L). Sens is shown in red in A′-F′. Expression of the constitutively active proteins encoded by *sSpi* (A), $EGFR^{\lambda top}$ (B) or Ras^{V12} (C) in wild-type clones induces excessive photoreceptor differentiation. In $Vps4^{3B1}$ clones, expression of *sSpi* (D) induces extra photoreceptor differentiation in wild-type cells surrounding the clone, but has no effect on the mutant cells. $EGFR^{\lambda top}$ expression in *Vps4* clones (E) does not rescue photoreceptor differentiation within the clones, but induces ectopic photoreceptors in surrounding wild-type tissue. In contrast, Ras^{V12} can induce photoreceptor differentiation within *Vps4* mutant clones (F). (G) A simplified diagram of the EGFR pathway showing that Vps4 acts upstream of Ras activation to promote photoreceptor differentiation and cell survival. (H-L) Activated Caspase 3 is stained in red. Control $EGFR^{\lambda top}$ (I) or Ras^{V12} (K) expressing clones do not induce cell death. The caspase activation observed in $Vps4^{3B1}$ clones (H) is rescued by *UAS-*Ras^{V12} (L) but not *UAS-*$EGFR^{\lambda top}$ (J).

mutant cells still showed increased photoreceptor differentiation in *Hrs* mutant eye discs, although loss of *Hrs* reduced photoreceptor differentiation in regions wild-type for *shi* (Miura et al., 2008) (Fig. 6B). Surprisingly, however, shi^{7C7} $Vps4^{3B1}$ double mutants showed the *Vps4* phenotype of missing photoreceptors and loss of *aos-lacZ* expression, even in a *Dronc* background in which cell death was prevented (Fig. 6C,D). *Vps4* was similarly epistatic to *shi* in clones expressing a dominant-negative thermo-sensitive allele of *shi* (Kitamoto, 2001) (Fig. 6E,F). As the molecular nature of the shi^{7C7} allele is unknown, we repeated the epistasis analysis with the previously described null allele shi^{FL54} (Windler and Bilder, 2010). shi^{FL54} $Vps4^{3B1}$ double mutant cells also failed to differentiate as photoreceptors, although removing *shi* prevented the endosomal accumulation of EGFR in these cells (Fig. 6G,H). These results are not consistent with an effect of *Vps4* only on internalized EGFR, and suggest that Vps4 can influence EGFR function prior to its endocytosis.

One possibility is that Vps4 might promote EGFR trafficking to the plasma membrane. To investigate this, we made use of the ovarian model system, in which the ligand-producing and responding cells can be distinguished. During egg chamber development, the EGFR ligand Gurken (Grk) is secreted from the oocyte and internalized into first posterior and later dorsal anterior follicle cells to specify their fates (Chang et al., 2008; Nilson and Schüpbach, 1998). This internalization requires both *shi* and *Egfr* (Chang et al., 2008), indicating that it is mediated by binding to surface EGFR and subsequent endocytosis. We found that clones of follicle cells mutant for *Vps4* were still able to internalize Grk, and in fact accumulated abnormally high levels of Grk that colocalized with internalized EGFR in punctate structures (Fig. 7A). EGFR

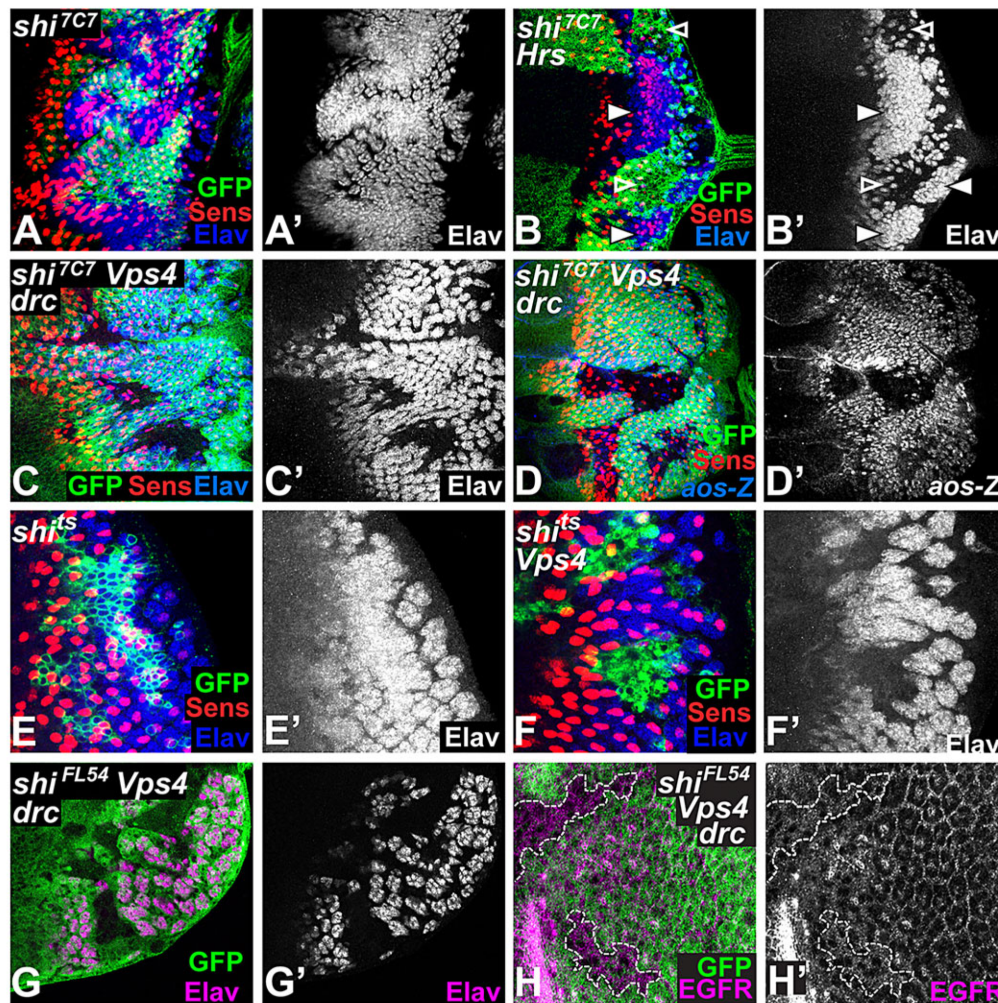

Fig. 6. *Vps4* blocks EGFR signaling upstream of Dynamin-mediated internalization. All panels show eye discs with clones marked by the absence of GFP (green) in A-D,G-H, or the presence of GFP (E,F). R8 is stained with Sens (red in A-F). Photoreceptors are stained with Elav (A′-C′,E′,F′, blue in A-C,E,F) or *aos-lacZ* is stained with anti-β-galactosidase (D′, blue in D). (A) shi^{7C7} clones differentiate excessive photoreceptors. (B) In an Hrs^{D28}/*Df(Hrs)* mutant disc in which photoreceptor differentiation is reduced (open arrowheads), shi^{7C7} clones still overproduce photoreceptors (filled arrowheads). (C,D) shi^{7C7} $Vps4^{3B1}$ double mutant clones in a $Dronc^{I29}$ (drc^{I29}) mutant background show loss of photoreceptors other than R8 and loss of *aos-lacZ* expression. (E,F) Clones expressing shi^{ts} in otherwise wild-type cells (E) or in $Vps4^{3B1}$ mutant cells (F). *Vps4* is still required for photoreceptor differentiation in the presence of this dominant-negative form of Shi. (G,H) shi^{FL54} $Vps4^{3B1}$ clones (outlined in H) in a $Dronc^{I29}$ mutant background are stained with Elav (G′, magenta in G) or EGFR (H′, magenta in H). Clones mutant for both *Vps4* and a *shi*-null allele lack photoreceptors but do not show punctate accumulation of EGFR.

must therefore be present on the surface of *Vps4* mutant follicle cells, allowing Grk reception. Surprisingly, *Vps4* was not required for EGFR signaling in these cells. In this system, EGFR signaling acts by preventing nuclear localization of the transcriptional repressor Capicua (Cic) (Astigarraga et al., 2007). Cic represses the expression of *mirr* (Atkey et al., 2006), which encodes a transcription factor that represses the ventral determinant *pipe* (Andreu et al., 2012). In dorsal follicle cells mutant for *Vps4*, a *mirr-lacZ* reporter was expressed normally, and no ectopic *pipe-lacZ* or nuclear localization of Cic was observed (Fig. 7B,C). The large clone size also indicated normal survival of these mutant cells. Notch signaling was likewise unaffected in *Vps4* mutant follicle cells. We saw no loss of its target gene *hindsight* (*hnt*; FlyBase – *peb*) or misexpression of *ct*, a gene repressed by Hnt (Sun and Deng, 2007), and mitotic cells marked by phosphorylated histone H3 were not observed in egg chambers older than stage 6 (Fig. 7D,E), despite strong intracellular accumulation of Notch protein (Fig. 7F). These results show that trapping of the EGFR with its ligand in the endocytic pathway is not sufficient to block EGFR signaling. Vps4 thus appears to promote EGFR signaling by a new non-endocytic and cell type-dependent mechanism, which could also be applicable to Notch and other receptors.

DISCUSSION

Vps4 affects multiple signaling pathways

We describe here a point mutation that specifically disrupts the function of *Drosophila Vps4*. A previous study used a deletion allele that unlike our mutation, could not be rescued by a wild-type *Vps4* construct, and thus might also disrupt one or both of the neighboring genes (Rodahl et al., 2009a). We confirmed previous findings that loss of *Vps4* results in JNK activation and apoptosis (Rodahl et al., 2009a). However, we found that blocking JNK activity did not prevent cell death or restore photoreceptor differentiation, indicating that JNK is not the primary driver of these effects of *Vps4* mutations. In contrast, activating EGFR signaling downstream of the receptor was sufficient to restore survival of R8 cells and differentiation of R1-R7, highlighting this pathway as central to the function of Vps4 in eye development.

As expected, given the known role for Vps4 and other ESCRT proteins in targeting receptors for lysosomal degradation (Hanson and Cashikar, 2012), EGFR and other receptors accumulate in enlarged endosomes in *Vps4* mutant cells. However, this accumulation has distinct effects on their activity; loss of *Vps4* reduces EGFR, Notch and Wg signaling, but increases Dpp signaling. The effect on Wg target genes is consistent with previous findings that endocytosis and MVBs promote Wnt signaling by sequestering Glycogen synthase kinase 3β, which would otherwise inhibit β-catenin (Dobrowolski et al., 2012; Seto and Bellen, 2006; Taelman et al., 2010). Dpp signaling is also thought to require endocytosis, because Smads are recruited to activated TGFβ family receptors by the endosomal protein Smad anchor for receptor activation (Sara) (Bennett and Alphey, 2002; Panopoulou et al., 2002; Tsukazaki et al., 1998). As Sara is present on early endosomes, signaling can be prolonged when progression

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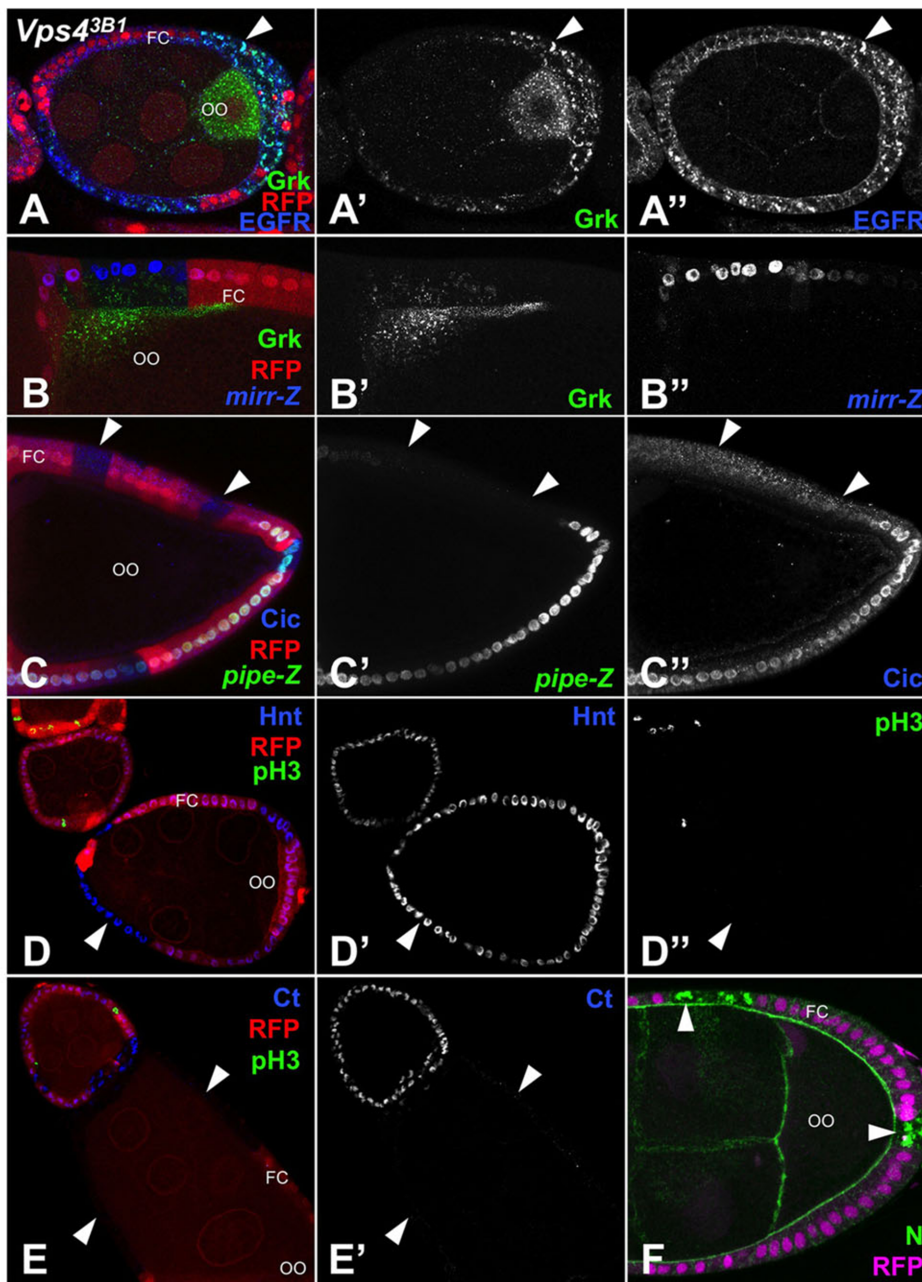

Fig. 7. Vps4 is not required for EGFR or Notch signaling in follicle cells. All panels show egg chambers with *Vps4*^{3B1} mutant clones in the follicle cells (FC) marked by the absence of RFP (red in A-E, magenta in F). OO, oocyte. Posterior is to the right. Arrowheads indicate representative clones. (A) Staining for Grk (A′, green in A) and EGFR (A″, blue in A) reveals that internalized Grk and EGFR accumulate and colocalize in mutant follicle cells. (B-C) Dorsal is up. (B) A mutant clone in follicle cells dorsal to the oocyte shows normal *mirr-lacZ* expression (B″, blue in B) and Grk internalization (B′, green in B). (C) Dorsal mutant follicle cell clones do not misexpress *pipe-lacZ* (C′, green in C) and Cic remains cytoplasmic, rather than localizing to the nucleus as seen in the ventral region (C″, blue in C). (D,E) Hnt (D′, blue in D) is expressed normally and there is no ectopic phospho-histone H3 (pH3) staining (D″, green in D,E) in *Vps4* clones. Ct (E′, blue in E) is not misexpressed in *Vps4* clones. Loss of Notch signaling would cause loss of Hnt and ectopic Ct and pH3 in egg chambers older than stage 6. (F) Notch (green) accumulates in puncta in *Vps4* clones.

of the receptor to late endosomes is blocked by loss of the ESCRT-II subunit Vps25 (Thompson et al., 2005) and perhaps also Vps4.

Vps4 has effects distinct from other ESCRT complex subunits

Vps4 mutants differ from previously described ESCRT mutations in their effects on Notch and EGFR signaling. Internalization of these receptors into the ILVs of MVBs segregates their intracellular domains from cytoplasmic effectors and should thus terminate signaling (Piper and Lehner, 2011). This model is consistent with the excessive Notch and EGFR signaling observed in eye and wing discs in the absence of many ESCRT proteins (Aoyama et al., 2013; Moberg et al., 2005; Rodahl et al., 2009b; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2009). This increased signaling has been attributed to the lack of lysosomal degradation of these receptors and their continued activity on the endosomal membrane.

Vps4 mutant cells instead show reduced expression of EGFR and Notch target genes, despite endosomal accumulation of the receptors. *Vps4*^{3B1} cells also fail to induce the non-autonomous overgrowth that results from misexpression of the Notch target gene *unpaired* (FlyBase – *outstretched*) in cells mutant for other ESCRT subunits (Thompson et al., 2005; Vaccari and Bilder, 2005). Notch cleavage and signaling is thought to occur in partially acidified endosomes and thus to require progression through the endocytic pathway (Schneider et al., 2013; Vaccari et al., 2010, 2008; Yan et al., 2009). Although EGFR signaling can occur at the plasma membrane (Brankatschk et al., 2012; Legent et al., 2012; Sousa et al., 2012), some studies suggest that receptor progression from early to late endosomes has a positive effect on signaling (Kim et al., 2007; Miura et al., 2008; Teis et al., 2006). Loss of *Vps4* might block endocytosis so late in the process of ILV formation that the intracellular domains of receptors are trapped in nascent ILV buds and no longer have access to the cytoplasm. Alternatively, an early

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block in receptor progression through endocytosis due to failure to recycle other ESCRT subunits (Babst et al., 1998) could reduce signaling.

Vps4 might contribute to EGFR activation

Another possible explanation for the effect of *Vps4* on EGFR signaling is suggested by our finding that loss of *shi* function does not restore EGFR signaling to *Vps4* mutant cells, even though it blocks receptor accumulation in the endocytic pathway. Dynamin is required to internalize mammalian EGFR by both clathrin-dependent and -independent mechanisms following its activation with all the ligands tested (Henriksen et al., 2013). In the absence of Dynamin, prolonged signaling of EGFR on the plasma membrane should be unaffected by defects in the late stages of endocytosis. The disruption of EGFR signaling by *Vps4* in *shi* mutant cells thus suggests that Vps4 has a role upstream of cell surface EGFR activation that is distinct from its function in endocytic MVBs. This model is supported by our observation that a constitutively active form of EGFR can partially rescue *Vps4* mutant cells (Fig. 5E). In addition, although *Vps4* affects the progression of EGFR and its ligand Grk, as well as Notch, through the endocytic pathway in follicle cells, signaling by both receptors remains intact in these cells, indicating that the two functions of Vps4 are separable.

The mechanism of this new effect of Vps4 is still unknown. As *Vps4* mutant clones accumulate high levels of intracellular EGFR, Vps4 is not required for EGFR transcription or translation. Although some studies have implicated ESCRT-III and Vps4 in recycling Ras and associated EGFR to the plasma membrane (Baldys and Raymond, 2009; Tu et al., 2011; Zheng et al., 2012), *Drosophila* Ras need not be associated with the membrane to function in photoreceptor recruitment (Sung et al., 2010), and a requirement for Vps4 in EGFR recycling would not explain its effect in the absence of Dynamin-mediated internalization. Vps4 has been reported to transport some newly synthesized proteins to the plasma membrane via endosomes (Futter et al., 1995; Yoshimori et al., 2000). However, the ability of *Vps4* mutant follicle cells to internalize Grk indicates that EGFR is present on the plasma membrane of these cells. It is possible that EGFR is trafficked by distinct routes in imaginal discs and in follicle cells. Grk is internalized through the apical domain of follicle cells (Tanentzapf et al., 2000), whereas active forms of Spi are localized basolaterally in discs (Steinhauer et al., 2013). A role for Vps4 in targeting the EGFR to the appropriate membrane domain would, however, not explain the requirement for Vps4 in cultured S2 cells, which lack apical-basal polarity. An alternative possibility is that Vps4 affects EGFR activation independently of its trafficking. Vps4 is involved in transporting cholesterol to the endoplasmic reticulum (Du et al., 2013), and could indirectly affect EGFR activation by altering the composition of membrane rafts (Balbis and Posner, 2010). Loss of *Vps4* in yeast was recently shown to result in the accumulation of misassembled nuclear pore complexes (Webster et al., 2014); although it seems unlikely that this would influence EGFR activation, it supports the existence of previously undescribed functions for Vps4. Further investigation of the effect of Vps4 on EGFR activity might define a new molecular mechanism of action for this versatile protein.

MATERIALS AND METHODS

Drosophila genetics

3B1 is an EMS-induced lethal allele of *Vps4* isolated previously (Legent et al., 2012). Using rescue by X chromosomal duplications followed by recombination with P-element markers (Zhai et al., 2003), we mapped it to a 0.35 cM interval in 16F1-5. The coding region of *Vps4* amplified from homozygous mutant larvae contained a missense mutation, E209K, that was absent from the *y,w, FRT19A* isogenic strain used for the mutagenesis. The rescuing duplication *Dp(1;3)JC153 (R1)* was obtained from Alberto Ferrus (Cajal Institute, Madrid, Spain). hep^{r75} (Glise et al., 1995) is a deletion of nucleotides 486 to 1346 that removes the start codon. Other stocks used were *wg-lacZ*^{P}, *puc-lacZ*^{E69}, *UAS-puc*^{2A}, *Hrs*^{D28}, *Df(Hrs)* [Exel6277], *aos-lacZ*^{W11}, *hh-lacZ*^{P30}, *mirr-lacZ*^{cre2} (Bloomington *Drosophila* Stock Center), *E(spl)mβ-CD2* (de Celis et al., 1998), *pipe-lacZ* (Andreu et al., 2012), *vps4*^{Δ7b} (Rodahl et al., 2009a), *Dronc*^{I29} (Xu et al., 2005), *shi*^{7C7} (Legent et al., 2012), *shi*^{FL54} (Windler and Bilder, 2010), UAS-*shi*^{ts} (Kitamoto, 2001), *TSG101*^{2} (Moberg et al., 2005), *UAS-rhomboid* (Lee et al., 2001), *UAS-sSpi* (Schweitzer et al., 1995), *UAS-EGFR*^{λtop} (Queenan et al., 1997) and *UAS-Ras*^{V12} (Karim and Rubin, 1998).

Stocks used to generate clones were: (1) *w, hsFLP*^{122}*, P[w+, ubi-GFP], FRT19A*, (2) *y,w, hsFLP*^{122}*, P[w+, ubi-RFP], FRT19A*, (3) *w, eyFLP1, tub-GAL80, FRT19A;; tub-GAL4, UAS-CD8::GFP/SM6-TM6B*, (4) *w, hsFLP*^{122}*, tub-GAL80, FRT19A;; tub-GAL4, UAS-CD8::GFP/SM6-TM6B*, (5) *w, eyFLP1, P[w+, ubi-GFP], FRT19A; Dronc*^{I29}*/TM6B*, (6) *w, hsFLP*^{122}*, P[w+, ubi-GFP], FRT19A; Dronc*^{I29}*/TM6B* and (7) *w, hsFLP*^{122}*, P[w+, ubi-RFP], FRT19A; Dronc*^{I29}*/TM6B*.

Molecular biology

The full-length *Vps4* (*CG6842*) coding region was amplified by PCR from the GH02678 EST clone (*Drosophila* Genomics Resource Center) using Pfu Turbo and cloned into *pUAST-HA* as an EcoRI-XhoI fragment to generate *pUAS-HA::Vps4*. Transgenic flies were generated by Genetic Services, Inc.

Immunohistochemistry

Third-instar eye and wing discs were dissected and stained as described previously (Legent and Treisman, 2008). Ovaries were stained as described previously (Miura et al., 2006). Antibodies used were: rabbit anti-β-galactosidase (1:5000, Cappel), chicken anti-GFP (1:500, Aves), mouse anti-HA (1:100, Covance), mouse anti-CD2 (1:50, Serotec), rabbit anti-active Caspase 3 (1:500, CM1, BD Pharmingen), rabbit anti-Ato (1:5000; Jarman et al., 1993), guinea pig anti-Sens (1:1000; Nolo et al., 2000), mouse anti-N^{ECD} (1:10), mouse anti-Dl^{ECD} (1:10), mouse anti-Wg (1:10), mouse anti-Ct (1:10), mouse anti-Grk (1:10), rat anti-Elav (1:100) (all Developmental Studies Hybridoma Bank, University of Iowa), rabbit anti-EGFR^{CTER} (1:500; Rodrigues et al., 2005), mouse anti-EGFR^{EXT} (1:200, Sigma E2906), rabbit anti-phospho-Smad (1:50, Cell Signaling 9516), guinea pig anti-Hrs (1:200; Lloyd et al., 2002), rabbit anti-Rab5 (1:50; Wucherpfennig et al., 2003), rabbit anti-Rab11 (1:1000; Satoh et al., 2005), chicken anti-Syx7 (1:500; Lu and Bilder, 2005), rabbit anti-Lva (1:5000; Sisson et al., 2000), guinea pig anti-Sec15 (1:500; Mehta et al., 2005), and rabbit anti-Cic (1:5000; Astigarraga et al., 2007). Secondary antibodies used were from Jackson Immunoresearch (1:200) or Molecular Probes (1:1000). Images were taken on a Leica SP5 confocal microscope. For *shi*^{ts} stainings, larvae were maintained at 31°C for 48 h prior to dissection.

Antibodies used for western blotting were mouse anti-diphosphorylated ERK (dpERK, 1:2500, Sigma M8159), rabbit anti-ERK (1:20,000, Cell Signaling 4695), mouse anti-Aos (1:100, Developmental Studies Hybridoma Bank), and mouse anti-β-Tubulin (1:3000, Covance MMS-410P) antibodies.

Cell culture

EGFR-expressing S2 (D2F) cells (Schweitzer et al., 1995) were maintained in Schneider's medium supplemented with 10% fetal calf serum and 150 µg/ml G418. Double-stranded RNAs (dsRNAs) were generated using the MEGAscript T7 and T3 Kits (Ambion) as described previously (Roignant et al., 2006) and 15 µg dsRNA were used to treat 10^6 cells per well for 5 days. EGFR expression was induced for 3 h with 60 µM Cu_2SO_4. For dpERK (pMAPK) western blots, the cells were treated with purified His-sSpiCS (Miura et al., 2006) for 10 min, and lysed as described previously (Miura et al., 2008). For Aos western blots, cells were serum-starved in medium containing dsRNA, and treated with purified His-sSpiCS for 16 h. Media were then harvested and the cells were lysed in ice-cold 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors

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(Roche). Western blotting was carried out as described previously (Miura et al., 2006). Total RNA was extracted from D2F cells using Trizol (Invitrogen). RT-PCR was performed on 1 µg of total RNA using the Invitrogen SuperScript First-Strand Kit. Primer sequences are available on request.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
K.L. and J.E.T. designed experiments; K.L., H.H.L. and J.E.T. performed experiments; K.L. and J.E.T. wrote the manuscript.

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