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Myopic acts in the endocytic pathway to enhance signaling by the *Drosophila* EGF receptor

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Endocytosis of activated receptors can control signaling levels by exposing the receptors to novel downstream molecules or by instigating their degradation. Epidermal growth factor receptor (EGFR) signaling has crucial roles in development and is misregulated in many cancers. We report here that Myopic, the *Drosophila* homolog of the Bro1-domain tyrosine phosphatase HD-PTP, promotes EGFR signaling in vivo and in cultured cells. *myopic* is not required in the presence of activated Ras or in the absence of the ubiquitin ligase Cbl, indicating that it acts on internalized EGFR, and its overexpression enhances the activity of an activated form of EGFR. Myopic is localized to intracellular vesicles adjacent to Rab5-containing early endosomes, and its absence results in the enlargement of endosomal compartments. Loss of Myopic prevents cleavage of the EGFR cytoplasmic domain, a process controlled by the endocytic regulators Cbl and Sprouty. We suggest that Myopic promotes EGFR signaling by mediating its progression through the endocytic pathway.

KEY WORDS: ESCRT complex, MAP kinase, HD-PTP (PTPN23), Bro1 domain, Photoreceptor

INTRODUCTION

The Epidermal growth factor receptor (EGFR) is required for cell differentiation and proliferation in numerous developmental systems (Shilo, 2003), and activation of the human EGFR homologs, ERBB1-4, is implicated in many cancers (Hynes and Lane, 2005). EGFR signaling events are terminated following removal of the receptor from the cell membrane by endocytosis. Ubiquitylation of EGFR by Cbl, an E3 ubiquitin ligase, initiates its internalization into clathrin-coated vesicles (Swaminathan and Tsygankov, 2006) and its transit through early and late endosomes, which differ by the exchange of Rab7 for Rab5 (Rink et al., 2005). EGFR can either return to the cell surface in Rab11-containing recycling endosomes, or reach the lysosome for degradation (Dikic, 2003; Seto et al., 2002). Delivery of receptors to the lysosome requires sorting from the limiting membrane of late endosomes into the internal vesicles of multivesicular bodies (MVBs) (Gruenberg and Stenmark, 2004), a process mediated by four endosomal sorting complexes required for transport (ESCRT-0, I, II and III) (Katzmann et al., 2002; Williams and Urbe, 2007). Ubiquitylated receptors are bound by Hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) in ESCRT-0, Tumor susceptibility gene 101 (Tsg101; also known as Erupted) in ESCRT-I and Vacuolar protein sorting 36 (Vps36) in ESCRT-II, and their deubiquitylation and internalization are coordinated by ESCRT-III (Williams and Urbe, 2007).

Genetic or pharmacological blocks of endocytosis prevent degradation of EGFR and other receptors. In *Drosophila*, *Hrs* mutations block MVB invagination, trapping receptor tyrosine kinases (RTKs) and other receptors on the outer membrane of the MVB, and sometimes leading to enhanced signaling (Jekely and Rorth, 2003; Lloyd et al., 2002; Rives et al., 2006; Seto and Bellen, 2006). Mutations in the ESCRT complex subunits *Tsg101* (ESCRT-I) and *Vps25* (ESCRT-II) cause overproliferation owing to the accumulation of mitogenic receptors such as Notch and Thickveins (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). In mammalian cells, loss of Hrs (also known as Hgs) or Tsg101 results in increased EGFR signaling (Bache et al., 2006; Razi and Futter, 2006). However, other studies have demonstrated a positive role for endocytosis in receptor signaling (Miaczynska et al., 2004; Seto and Bellen, 2006; Teis and Huber, 2003). Mutations affecting the *Drosophila* trafficking protein Lethal giant discs dramatically increase Notch signaling only in the presence of Hrs, indicating that signaling is maximized at a specific point in the endocytic process (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006). Wingless (Wg) signaling is enhanced by internalization into endosomes, where it colocalizes with downstream signaling molecules (Seto and Bellen, 2006). In mammalian cells, EGFR encounters the scaffolding proteins Mek1 partner (Mp1) and p14, which are required for maximal phosphorylation of the downstream component mitogen-activated protein kinase (MAPK), only on endosomes (Pullikuth et al., 2005; Teis et al., 2006).

Here we describe the characterization of the novel *Drosophila* gene *myopic* (*mop*). Loss of *mop* affects EGFR-dependent processes in eye and embryonic development, and reduces MAPK phosphorylation by activated EGFR in cultured cells. Mop acts upstream of Ras activation to promote the function of activated, internalized EGFR. Mop is homologous to human HD-PTP (PTPN23 – Human Gene Nomenclature Database) (Toyooka et al., 2000), which contains a Bro1 domain that is able to bind the ESCRT-III complex component SNF7 (CHMP4B – Human Gene Nomenclature Database) (Ichioka et al., 2007; Kim et al., 2005) and a tyrosine phosphatase domain. Mop is present on intracellular vesicles, and cells lacking *mop* have enlarged endosomes and reduced cleavage of the EGFR cytoplasmic domain. We propose that Mop potentiates EGFR signaling by enhancing its progression through endocytosis. Consistent with this hypothesis, we find that components of the ESCRT-0 and ESCRT-I complexes are also required for EGFR signaling in *Drosophila* cells.

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DEVELOPMENT

MATERIALS AND METHODS

Fly stocks and genetics

Four alleles of *mop* were isolated in a mosaic screen for genes required for photoreceptor differentiation (Janody et al., 2004). *mop* was mapped by meiotic recombination with P(w^+) elements (Zhai et al., 2003) to a 30 kb region containing five predicted genes: *CG9384*, *CG17173*, *CG9311*, *CG5295* (*bmm*) and *CG13472*. The coding regions of these genes were amplified by PCR from homozygous *mop* mutant embryos. Changes were found only in *CG9311*, which had stop codons at Q351 in mop^{T612}, Q538 in mop^{T862} and at Q1698 in mop^{T482}. Other strains used were UAS-$EGFR^{\lambda top}$ (Queenan et al., 1997), Cbl^{F165} (Pai et al., 2000), Hrs^{D28} (Lloyd et al., 2002), $sty^{\Delta5}$, UAS-Ras^{V12}, $aos^{\Delta7}$, *aos-lacZ*^{W11}, *dpp-lacZ{BS3.0}*, *Dll-lacZ*^{01092}, *ap*-GAL4, *Actin*>CD2>GAL4, and *Df(2L)Exel6277* (FlyBase). Stocks used to make clones were: (1) *ey*FLP1; FRT80, Ubi-GFP; (2) *ey*FLP1; FRT80, *M(3)67C*, Ubi-GFP/TM6B; (3) *hs*FLP122; FRT80, Ubi-GFP; (4) *hs*FLP122; FRT80, *M(3)67C*, Ubi-GFP/TM6B; (5) FRT2A, mop^{T612}/TM6B; (6) *hs*FLP122; FRT2A, P(ovo^D)/TM3; and (7) *ey*FLP1, UAS-GFP; *tub*-GAL4; FRT80, *tub*-GAL80. *mop* mutant clones in *Hrs* mutant eye discs were generated by crossing Hrs^{D28}, *ey*FLP; FRT80, mop^{T612}/SM6-TM6B to *Df(2L)Exel6277*; FRT80, Ubi-GFP/SM6-TM6B. UAS-*mop* was made by cloning a *Bgl*II fragment from the full-length cDNA SD03094 (*Drosophila* Genomics Resource Center) into pUAST. UAS-*mopCS* was made by PCR, using primers that changed C1728 to S and also introduced a *Kpn*I site by changing S1732 to T. UAS-FlagMop was generated by PCR amplification of an N-terminal *Eco*RI/*Xho*I fragment using primers that introduced an N-terminal Flag tag.

Immunohistochemistry and western blotting

Staining of eye and wing discs with antibodies or X-Gal was performed as described (Lee et al., 2001). Antibodies used were rat anti-Elav (1:100), mouse anti-Cyclin B (1:50), mouse anti-Cut (1:1), mouse anti-Wg (1:5) (Developmental Studies Hybridoma Bank), guinea pig anti-Sens (1:1000) (Nolo et al., 2000), rabbit anti-Ato (1:5000) (Jarman et al., 1995), rabbit anti-CM1 (anti-active caspase 3) (1:500; BD Pharmingen), rabbit anti-β-galactosidase (1:5000; Cappel), rabbit anti-GFP (1:1000; Molecular Probes), mouse anti-dpERK (Rolled – FlyBase) (1:250; Sigma), rat anti-Ci (1:1) (Motzny and Holmgren, 1995), guinea pig anti-Hrs (1:200) (Lloyd et al., 2002), guinea pig anti-Dor (1:200) (Sevrioukov et al., 1999), guinea pig anti-Spinster (1:250) (Sweeney and Davis, 2002), rabbit anti-Rab11 (1:1000) (Satoh et al., 2005), mouse anti-Flag (1:500; Sigma), mouse anti-Mop (1:100; Abcam) and rabbit anti-EGFR (1:500) (Rodrigues et al., 2005). Embryos were stained with rabbit anti-Slam (1:1000) after heat fixation as described (Stein et al., 2002). TOTO-3 dye was used at 1:3000 for 15 minutes, on embryos treated with 100 μg/ml RNase for 30 minutes before the secondary antibody. In situ hybridization was performed as described (Roignant et al., 2006), using sense and antisense probes transcribed from the *mop* cDNA SD03094, or an antisense probe transcribed from a 1.5 kb PCR product encompassing the *hkb* coding region. UAS-GFPRab5, UAS-GFPRab7, UAS-GFPRab11 and UAS-lgp120-GFP were a gift from Henry Chang (Chang et al., 2004). S2R+ cells were fixed in PBS containing 4% formaldehyde and stained as described (Miura et al., 2006). Images were captured on a Zeiss LSM 510 confocal microscope. Western blots were performed as described (Miura et al., 2006). Antibodies used were mouse anti-dpERK (1:2500; Sigma), mouse anti-ERK (1:20,000; Sigma), mouse anti-Tubulin (1:1000; Covance), rabbit anti-EGFR (1:10,000) (Lesokhin et al., 1999), mouse anti-Mop (1:1000; Abcam) and mouse anti-GFP (1:300; Santa Cruz).

Cell culture and RNAi

S2 and S2R+ cells were maintained in Schneider's medium supplemented with 10% fetal calf serum; EGFR-expressing S2 (D2F) cells (Schweitzer et al., 1995) were additionally supplemented with 150 μg/ml G418 and sSpiCS-expressing cells (Miura et al., 2006) with 150 μg/ml hygromycin. Cells were transfected using Effectene (Qiagen). UAS plasmids were cotransfected with *Actin*-GAL4. UAS-HACblL was cloned by PCR amplification of a cDNA representing the longer Cbl isoform. Double-stranded RNAs (dsRNAs) were generated using the MEGAscript T7 and T3 Kit (Ambion) as described (Roignant et al., 2006) and 15 μg dsRNA were used to treat 10^6 cells/well. S2R+ cells were transfected with *Actin*-GAL4, UAS-GFP, and UAS-EGFRλtop (Queenan et al., 1997) 1 day after dsRNA incubation. Cells were harvested after 2 days and lysed in ice-cold 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, protease inhibitors (Roche). D2F cells treated with dsRNA for 4 days were serum-starved overnight in dsRNA. EGFR expression was induced for 3 hours with 60 μM Cu_2SO_4, and the cells were transferred to sSpiCS-conditioned medium prepared by growing cells stably expressing sSpiCS in serum-free medium containing 500 μM Cu_2SO_4 for 4 days. Cells were lysed in RIPA buffer (Schweitzer et al., 1995). 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.

Internalization of Alexa-labeled Spitz

Cells stably expressing pMT-sSpiCSHis were grown to 5×10^6 cells/ml in serum-free medium and induced for 4 days with 500 μM Cu_2SO_4. Medium was collected and diafiltered against 150 mM NaCl, 25 mM HEPES (pH 8). sSpiCSHis was purified using the Ni-NTA Fast Start Kit (Qiagen), concentrated using Centricon columns (Millipore), diafiltered again, and labeled using an Alexa Fluor 546 Protein Labeling Kit (Molecular Probes). D2F cells were incubated with dsRNA for 4 days, serum-starved overnight with dsRNA, and EGFR expression was induced for 16 hours with 500 μM Cu_2SO_4. Cells were incubated with 100 nM Alexa-labeled sSpiCS in 1% BSA in PBS on ice for 30 minutes, washed three times with 1% BSA in PBS, incubated in serum-free medium with 75 nM Lysotracker (Molecular Probes) at room temperature and imaged by confocal microscopy. Vesicles containing Spi were scored as negative, weakly or strongly stained with Lysotracker by two independent observers.

RESULTS

mop is required for EGFR signaling during eye development

Photoreceptor differentiation in the *Drosophila* eye disc is driven by the secreted protein Hedgehog (Hh) (Heberlein and Moses, 1995). Hh induces the transcription factor Atonal (Ato), which promotes the differentiation of R8 photoreceptors posterior to the morphogenetic furrow (Dominguez, 1999; Jarman et al., 1995). R8 then secretes the EGFR ligand Spitz (Spi), which recruits photoreceptors R1-7 into each cluster (Freeman, 1997). In a screen for genes required for photoreceptor differentiation (Janody et al., 2004), we isolated four ethyl methanesulfonate (EMS)-induced alleles of a previously undescribed gene that we have named *myopic* (*mop*). In *mop* mutant clones, fewer photoreceptors, as visualized by staining with the neuronal nuclear marker Elav, were present (Fig. 1A,A′). However, R8 differentiation appeared almost normal as judged by expression of the markers Ato and Senseless (Sens) (Frankfort et al., 2001) (Fig. 1B-B″,C-C″), suggesting that the primary defect is in the recruitment of R1-7 through EGFR signaling.

EGFR signaling is also required in the eye disc for cell survival and cell cycle arrest. Mutations in EGFR pathway components increase cell death posterior to the morphogenetic furrow (Baonza et al., 2002; Roignant et al., 2006; Yang and Baker, 2003). We observed activated Caspase 3 staining, indicative of apoptotic cells, in posterior *mop* mutant clones (Fig. 1D,D′). Loss of EGFR signaling also prevents the R2-R5 precursors from arresting in G1 phase (Roignant et al., 2006; Yang and Baker, 2003). Expression of the G2-phase marker Cyclin B was increased in *mop* mutant clones, indicating that more cells re-entered the cell cycle (Fig. 1E,E′). Finally, we examined EGFR signaling directly by looking at phosphorylation of the downstream component MAPK using a phospho-specific antibody (Gabay et al., 1997b). Phospho-MAPK staining was reduced in *mop* mutant clones (Fig. 1F,F′), confirming a role for *mop* in EGFR signaling.

DEVELOPMENT

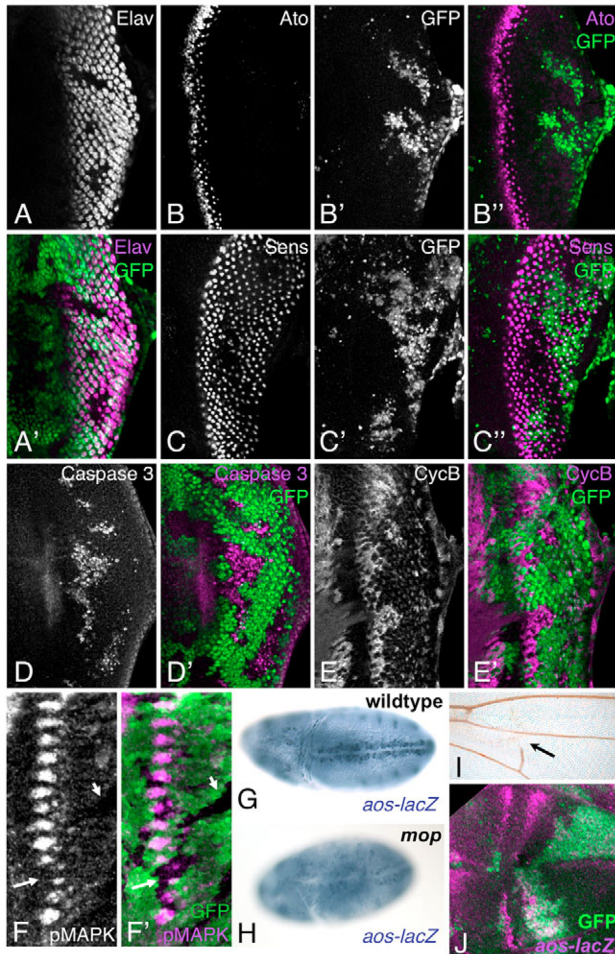

Fig. 1. *mop* is required for EGFR signaling. (**A-F′**) Third instar *Drosophila* eye discs. (A,A′) mop^{T612} mutant clones marked by the absence of GFP (green in A′). Photoreceptors are stained with anti-Elav (A, magenta in A′). (B-B″,C-C″) Eye discs with large mop^{T612} mutant clones generated in a *Minute* background and marked by the absence of GFP (B′,C′, green in B″,C″). R8 photoreceptors are stained with anti-Ato (B, magenta in B″) or anti-Sens (C, magenta in C″). *mop* has little effect on R8 differentiation. (D-F′) mop^{T612} mutant clones marked by the absence of GFP (green in D′,E′,F′). Activated Caspase 3 staining (D, magenta in D′) marks apoptotic cells and Cyclin B staining (E, magenta in E′) marks cells in G2 or M phase. Posterior *mop* mutant clones contain reduced numbers of photoreceptors and show increased cell death and cell cycle re-entry. Phospho-MAPK staining (F, magenta in F′) is reduced in *mop* mutant clones in the morphogenetic furrow (long arrow) and posteriorly (short arrow). (**G**,**H**) Embryos stained with anti-β-galactosidase reflecting *aos-lacZ* expression. (G) Wild type; (H) maternal/zygotic *mop* mutant. *aos* expression is strongly reduced in the absence of *mop*. (**I**) An adult wing containing mop^{T612} mutant clones shows loss of wing vein material (arrow). (**J**) A third instar wing disc with mop^{T612} clones made in a *Minute* background and marked by the absence of GFP (green), stained with anti-β-galactosidase reflecting *aos-lacZ* expression (magenta).

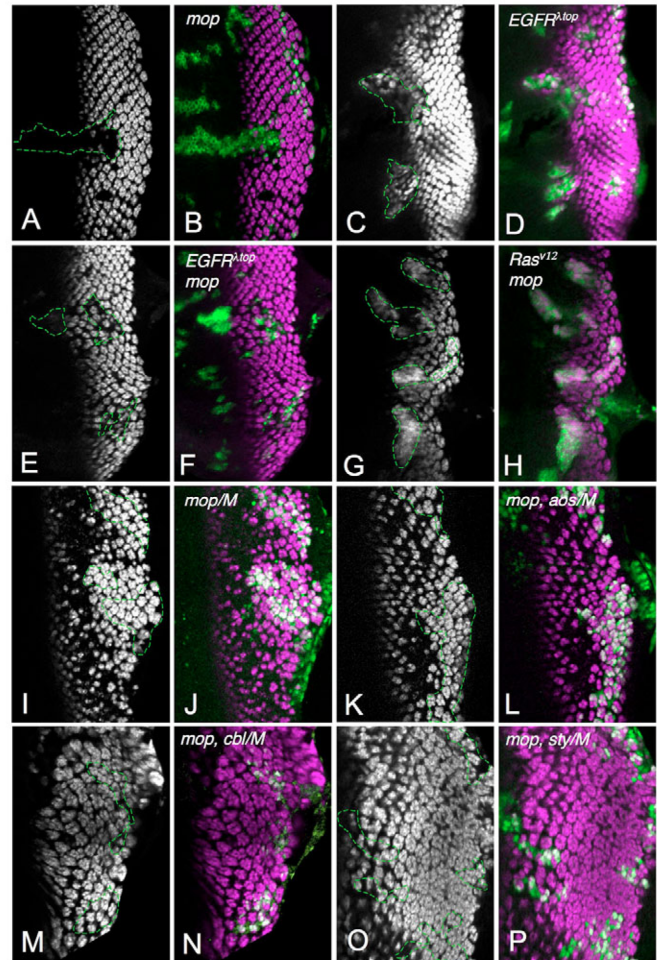

Fig. 2. *mop* acts on internalized EGFR. All panels show third instar *Drosophila* eye discs. Photoreceptors are stained with anti-Elav (A,C,E,G,I,K,M,O; magenta in B,D,F,H,J,L,N,P). (**A**,**B**) mop^{T482} mutant clones are positively marked by GFP expression (green in B, outlined in A). (**C**,**D**) Clones expressing *EGFRλtop* are positively marked by GFP expression (green in D, outlined in C). (**E**,**F**) mop^{T612} mutant clones expressing *EGFRλtop* are positively marked by GFP expression (green in F, outlined in E). (**G**,**H**) mop^{T612} mutant clones expressing Ras^{v12} are positively marked by GFP expression (green in H, outlined in G). (**I-P**) Large clones generated in a *Minute* background are marked by the absence of GFP (green in J,L,N,P, outlined in I,K,M,O). (I,J) mop^{T612}; (K,L) mop^{T612} $aos^{\Delta 7}$; (M,N) mop^{T612} Cbl^{F165}; (O,P) mop^{T612} $sty^{\Delta 5}$. Although *EGFRλtop* induces photoreceptor differentiation in a wild-type background, it does not rescue *mop* mutant clones; removing *aos* also fails to rescue. Photoreceptor differentiation can be restored to *mop* mutant clones by expressing Ras^{v12} or removing *Cbl* or *sty*.

EGFR signaling is also active at the embryonic midline and in the wing vein primordia, where it turns on expression of the target gene *argos* (*aos*) (Gabay et al., 1997a; Golembo et al., 1996; Guichard et al., 1999). In embryos lacking maternal and zygotic *mop*, midline *aos* expression was strongly reduced (Fig. 1G,H). Adult wings that contained *mop* mutant clones had missing wing veins (Fig. 1I), although *aos* was still detectable in *mop* clones in the wing disc (Fig. 1J). We also examined signaling by another RTK, Torso. Torso specifies the termini of the embryo by inducing target genes that include *huckebein* (*hkb*) (Ghiglione et al., 1999). *hkb* was expressed normally in embryos derived from *mop* mutant germline clones (see Fig. S1A,B in the supplementary material); *mop* is thus not essential for Torso signaling and might be specific to the EGFR pathway.

mop is not required in the absence of Cbl

To determine where Mop functions within the EGFR pathway, we attempted to rescue *mop* mutant clones by activating other components of the pathway. Although expression of an activated

DEVELOPMENT

form of EGFR (Queenan et al., 1997) in wild-type cells caused ectopic photoreceptor differentiation, its expression in *mop* mutant cells did not rescue the loss of photoreceptors (Fig. 2C-F). However, an activated form of the small GTPase Ras (Karim and Rubin, 1998) was able to induce excessive photoreceptor differentiation when expressed in *mop* mutant cells (Fig. 2G-H). These results place Mop function downstream of EGFR activation but upstream of Ras. In agreement with an intracellular action of Mop, removal of Aos, which inhibits pathway activation extracellularly by binding to Spi (Klein et al., 2004), did not significantly restore photoreceptor differentiation in the absence of *mop* (Fig. 2I-L).

To determine the position of Mop more precisely, we used a negative regulator of the pathway that also acts between EGFR and Ras. Cbl is an E3 ubiquitin ligase required for internalization and degradation of EGFR (Pai et al., 2000; Swaminathan and Tsygankov, 2006). Although loss of *Cbl* only mildly increases photoreceptor differentiation (Wang et al., 2008), the photoreceptor loss observed in *mop* mutant clones was restored in clones doubly mutant for *mop* and *Cbl* (Fig. 2M,N). This result indicates that the absence of photoreceptors in *mop* mutant clones is specifically due to reduced signaling by EGFR or other RTKs regulated by Cbl, and that *mop* is only required for the activity of EGFR molecules that have been internalized through Cbl activity.

mop encodes a novel endosomal protein

We used recombination with molecularly characterized P(w^+) insertions (Zhai et al., 2003) to map *mop* to a region containing five predicted genes. Genomic DNA isolated from three of our *mop* alleles contained nonsense mutations in one of these genes, *CG9311*, that were not present in the isogenic strain used for the screen (Fig. 3A). To confirm that *mop* corresponded to *CG9311*, we showed that expression of a *CG9311* transgene in *mop* mutant clones was sufficient to rescue photoreceptor differentiation (Fig. 3C,D). In situ hybridization showed that *mop* transcripts were present ubiquitously in early embryos and imaginal discs, and at high levels in the nervous system and gut at later embryonic stages (see Fig. S2A-F in the supplementary material).

To determine whether Mop could activate the EGFR pathway, we expressed UAS-*mop* in the dorsal compartment of the wing disc using *apterous* (*ap*)-GAL4 and examined the expression of the EGFR target gene *aos*. Expression of Mop only very weakly activated *aos* expression (Fig. 3H), whereas a constitutively active form of EGFR induced strong *aos* expression (Fig. 3I). Coexpression of Mop potentiated the effect of activated EGFR, increasing the level of *aos* expression and inducing overgrowth of the dorsal compartment of the disc (Fig. 3J). Similarly, coexpression of Mop enhanced the ability of activated EGFR to induce ectopic photoreceptor differentiation in the eye disc (data not shown). We conclude that Mop does not itself activate EGFR, but the maximal activity of the activated receptor depends on the level of Mop expression.

mop encodes a protein of 1833 amino acids with a Bro1 domain (Kim et al., 2005) at its N-terminus and a region of homology to tyrosine phosphatases at its C-terminus (Fig. 3A). However, some amino acids thought to be crucial for phosphatase activity (Andersen et al., 2001) are not conserved in the Mop tyrosine phosphatase domain (Fig. 3B). We tested whether phosphatase activity was required for Mop function by mutating the catalytic cysteine in the predicted active site to a serine (Fig. 3B). Expression of this transgene (MopCS) rescued photoreceptor differentiation in *mop* mutant clones as effectively as the wild-type Mop transgene (Fig. 3E,F), suggesting that tyrosine phosphatase activity is not essential for Mop function in the eye disc.

The Bro1 domain of yeast Bro1 is sufficient to mediate endosomal localization (Kim et al., 2005), and Bro1-domain proteins are important for endocytic trafficking (Odorizzi, 2006). We

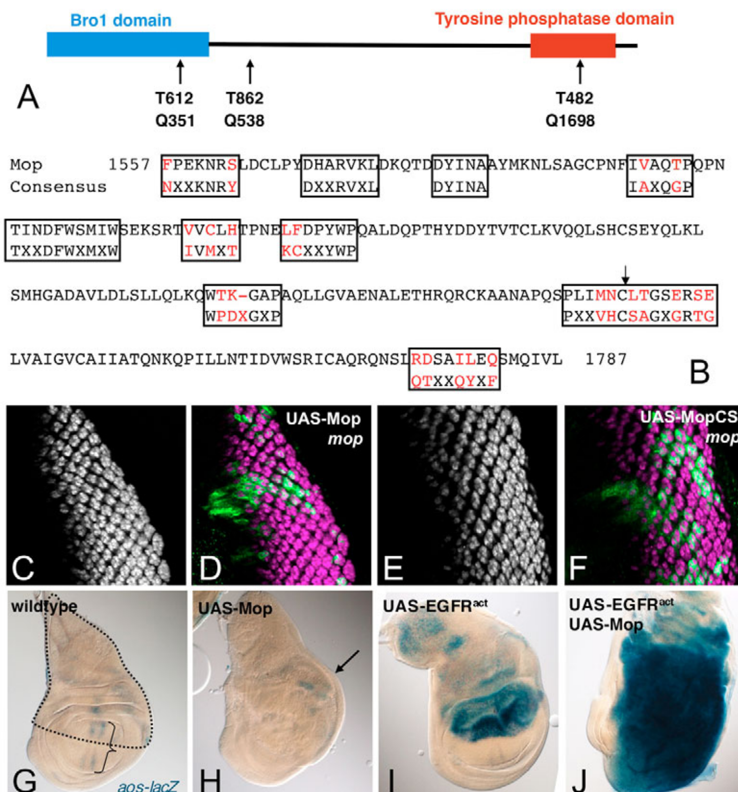

Fig. 3. Structure and expression of the *Drosophila* Mop protein. (**A**) Mop contains regions of homology to Bro1 and to tyrosine phosphatases. The positions of stop codons introduced by three *mop* mutant alleles are indicated. (**B**) Comparison of the Mop tyrosine phosphatase domain with the consensus sequences for the ten functional motifs defined for tyrosine phosphatases (boxed). Amino acids that differ from the consensus sequence are in red; the arrow indicates the predicted active site cysteine. (**C-F**) Third instar eye imaginal discs with clones positively marked by GFP expression (green in D,F) stained with anti-Elav (C,E, magenta in D,F). (C,D) mop^{T482} clones expressing a wild-type UAS-*mop* transgene; (E,F) mop^{T482} clones expressing a phosphatase-dead transgene (UAS-*mopCS*). Both transgenes fully rescue photoreceptor differentiation. (**G-J**) Third instar wing imaginal discs expressing *aos-lacZ* and *ap*-GAL4 and stained overnight with X-Gal. *ap*-GAL4 drives expression in the dorsal compartment (outlined in G). (G) Wild type; *aos* is weakly expressed in the wing vein primordia (bracket). (H) UAS-FlagMop; (I) UAS-EGFRλtop; (J) UAS-FlagMop and UAS-EGFRλtop. Mop overexpression gave very weak ectopic *aos* expression (arrow, H), but strongly enhanced the effect of activated EGFR.

therefore examined the subcellular localization of Mop. Using an antibody generated by the UT Southwestern Genomic Immunization Project that specifically recognized Mop on western blots (see Fig. 5D), we observed punctate intracellular localization of the endogenous protein in *Drosophila* S2R+ cells (Fig. 4A). Since

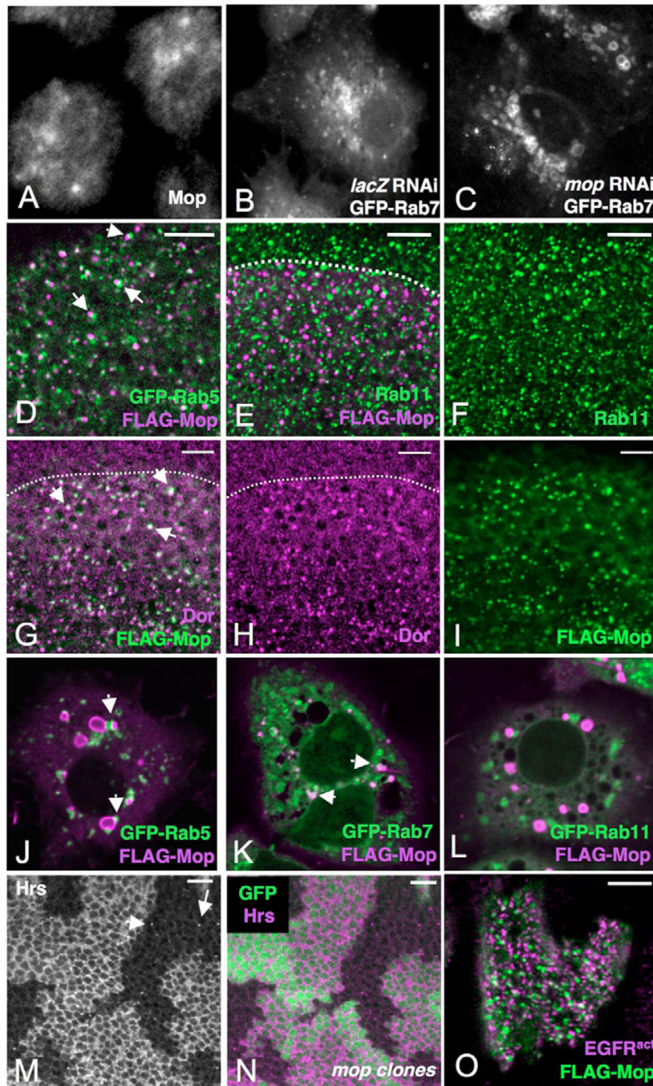

Fig. 4. Mop is an endosomal protein. (**A**) *Drosophila* S2R+ cells stained for endogenous Mop. (**B**,**C**) GFP-Rab7 fluorescence in live S2R+ cells treated with dsRNA targeting *lacZ* (B) or *mop* (C). Mop depletion causes enlargement of Rab7-containing endosomes. (**D-I**) Wing discs expressing UAS-FlagMop with *ap*-GAL4 and stained for Flag (magenta in D,E; green in G,I), coexpressed Rab5-GFP (green in D), Rab11 (green in E,F), or Dor (magenta in G,H). (**J-L**) S2R+ cells expressing UAS-FlagMop and UAS-GFPRab5 (J), UAS-GFPRab7 (K) or UAS-GFPRab11 (L) with *Actin*-GAL4. Flag staining is shown in magenta and GFP in green. Mop is present on vesicles that are adjacent to Rab5-containing vesicles (arrowheads in D,J), shows partial colocalization with Dor and Rab7 (arrowheads in G,K), and does not colocalize with Rab11. (**M**,**N**) Wing discs with mop^{T612} clones marked by the absence of GFP (green in N), stained with anti-Hrs (M, magenta in N). Hrs shows reduced levels and punctate localization (arrows, M) in *mop* mutant clones. (**O**) An eye disc with a clone of cells expressing UAS-FlagMop and UAS-EGFRλtop, stained with anti-Flag (green) and anti-EGFR (magenta). Most of the activated EGFR is present in vesicles that do not contain Mop. Scale bars: 10 μm.

endogenous Mop levels were too low to obtain high-resolution images, we generated a transgene expressing an N-terminally Flag-tagged Mop protein, which was able to rescue photoreceptor differentiation in *mop* mutant clones (see Fig. S2G,H in the supplementary material). Flag-Mop was located at the membrane of intracellular vesicles in imaginal discs and S2R+ cells (Fig. 4D-L). These vesicles were often adjacent to vesicles expressing the early endosomal marker GFP-Rab5 (Fig. 4D,J). We observed some colocalization of Mop with the late endosomal markers GFP-Rab7, Deep orange (Dor) (Sevrioukov et al., 1999; Sriram et al., 2003) and Hrs, though these markers appeared more punctate in Mop-overexpressing cells (Fig. 4G-I,K and data not shown). However, we saw no colocalization of Mop with the recycling endosome marker Rab11 or with the lysosomal markers GFP-lgp120 and Spinster (Chang et al., 2004; Satoh et al., 2005; Sweeney and Davis, 2002) (Fig. 4E,F,L and data not shown).

Removal of proteins required for progression through endocytosis often results in the enlargement of specific endocytic compartments (Raymond et al., 1992). We found that S2R+ cells in which *mop* was depleted by RNA interference (RNAi) showed an enlargement of endosomes labeled by GFP-Rab7 (Fig. 4B,C). Loss of *mop* also had a striking effect on Hrs distribution in vivo: Hrs levels appeared strongly reduced in *mop* mutant clones, and the remaining Hrs protein was punctate rather than diffusely localized (Fig. 4M,N; see Fig. S3A,B in the supplementary material). These observations suggest that Mop has an essential role in the endocytic pathway. Although loss of *Cbl* was able to rescue the EGFR signaling defects in *mop* mutant cells (Fig. 2M,N), it did not rescue the endocytic defects that result in Hrs mislocalization (see Fig. S3A-F in the supplementary material), supporting the model that rescue is observed because EGFR remains on the cell surface.

In the early *Drosophila* embryo, cells are formed by invagination of membranes between the nuclei; this process requires apical-basal transfer of membrane through endocytosis and recycling (Lecuit, 2004). Injection of embryos with dominant-negative Rab5 or Rab11 causes defective membrane invagination and loss of nuclei from the embryo cortex (Pelissier et al., 2003). Embryos derived from *mop* mutant germline clones showed similar cellularization defects. Membrane invagination was irregular and some nuclei lost their association with the cortex (see Fig. S1C-F in the supplementary material), consistent with a role for Mop in endocytosis.

Mop is required for EGFR processing

The presence of Mop on intracellular vesicles and its effect on endosome size suggested that Mop might enhance EGFR signaling by controlling its endocytic trafficking. However, Mop does not prevent EGFR protein degradation, as *mop* mutant clones in the eye disc showed a slight increase in EGFR levels (Fig. 5A-C). To look for other effects on EGFR we used cultured S2 cells, in which Mop levels could be strongly reduced by RNAi (Fig. 5D,G). We first tested whether *mop* was required for EGFR signaling in these cells, using MAPK phosphorylation to monitor EGFR activity (Gabay et al., 1997b). Treatment of an S2 cell line that stably expresses EGFR [D2F (Schweitzer et al., 1995)] with media conditioned by cells expressing Spi (Miura et al., 2006) induced significant MAPK phosphorylation after 30 minutes. This phosphorylation was strongly reduced in cells treated with *mop* RNAi (Fig. 5E), confirming a requirement for Mop in EGFR signal transduction. In D2F cells stimulated with fluorescently labeled purified Spi, knocking down *mop* by RNAi did not prevent Spi uptake into intracellular vesicles (see Fig. S4 in the supplementary material); thus Mop does not affect the cell surface expression of EGFR or its

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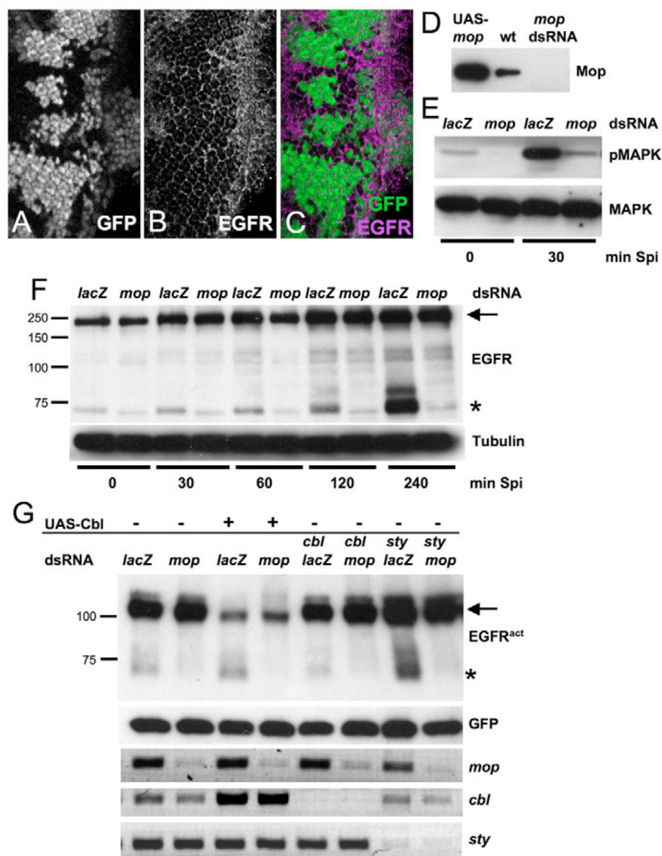

Fig. 5. Mop is required for MAPK phosphorylation and EGFR cleavage. (**A-C**) *Drosophila* eye disc with mop^{T612} mutant clones marked by the absence of GFP (A, green in C) and stained with anti-EGFR (B, magenta in C). EGFR levels are slightly increased in *mop* mutant clones. (**D**) Western blot with Mop antibody of extracts from S2 cells transfected with *Actin*-GAL4 and UAS-*mop*, untreated (wt) or treated with *mop* dsRNA. *mop* is expressed in S2 cells and its levels can be significantly reduced by RNAi. (**E**) D2F cells were treated with *lacZ* or *mop* dsRNA and incubated with sSpi-conditioned media for 0 or 30 minutes. Protein lysates were blotted with antibodies to diphospho-MAPK and total MAPK. *mop* dsRNA treatment resulted in a decrease in MAPK phosphorylation. (**F**) Western blot with anti-EGFR of lysates from D2F cells treated with *lacZ* or *mop* dsRNA and incubated with sSpi-conditioned media for the indicated times. Cells treated with *mop* dsRNA failed to accumulate a faster-migrating band recognized by the EGFR antibody (asterisk). The position of full-length EGFR is indicated by an arrow. (**G**) S2R+ cells were treated with *lacZ*, *mop*, *Cbl* and/or *sty* dsRNA as indicated, and transfected with *Actin*-GAL4, UAS-GFP and UAS-*EGFRλtop*, and UAS-*CblL* in the indicated lanes. Protein lysates were blotted with antibodies to EGFR and GFP (transfection control). A smaller band recognized by the EGFR antibody that is the same size as the smaller band in F, is indicated by an asterisk; the arrow indicates full-length EGFRλtop. The proportion of the smaller band is increased by Cbl cotransfection or *sty* RNAi and decreased by *mop* RNAi. The lower three panels show RT-PCR quantification of *mop*, *Cbl* and *sty* mRNA, demonstrating the efficiency of the RNAi treatment.

ability to bind and internalize Spi. However, *mop* RNAi treatment did alter the colocalization of fluorescent Spi with Lysotracker, a dye that detects lysosomes by their low pH. The proportion of Spi-containing vesicles with strong Lysotracker staining 3-4 hours after Spi treatment was reduced in *mop*-depleted cells (see Fig. S4A-G in the supplementary material). *mop* depletion increased the proportion of Spi-positive vesicles showing weak Lysotracker accumulation (see Fig. S4G in the supplementary material), suggesting that Spi is retained in endosomes that have begun the process of acidification. These data are consistent with a reduction in EGFR traffic to the lysosome in the absence of Mop.

When we examined EGFR by western blotting in D2F cells following Spi treatment, we observed the progressive accumulation of a faster-migrating band recognized by an antibody generated against the extreme C-terminus of EGFR (Lesokhin et al., 1999) (Fig. 5F). This band is the appropriate size (60 kDa) to be the cytoplasmic domain of the receptor, suggesting that it is produced by juxtamembrane cleavage. The same size band was observed in S2R+ cells transfected with an activated form of EGFR (λtop) (Fig. 5G), although this form has a smaller, unrelated extracellular domain derived from the lambda repressor (Queenan et al., 1997). The relative abundance of the smaller band was increased by cotransfection with a Cbl expression construct and was reduced by Cbl depletion (Fig. 5G), consistent with cleavage occurring in the endocytic pathway. The appearance of this smaller band was prevented by *mop* depletion, both in D2F cells treated with Spi and in S2R+ cells transfected with EGFRλtop (Fig. 5F,G), suggesting that *mop* is required for EGFR to reach the compartment in which it is cleaved.

Progression through endocytosis enhances EGFR signaling

Receptor signaling terminates when invagination of the MVB outer membrane traps the cytoplasmic domains of receptors inside the inner vesicles. Hrs acts at the first step in this process, and *Hrs* mutants have been reported to result in enhanced EGFR signaling in the embryo and ovary (Jekely and Rorth, 2003; Jekely et al., 2005; Lloyd et al., 2002). We therefore examined the role of Hrs in EGFR signaling in imaginal discs. Surprisingly, *Hrs* mutant eye discs showed a loss of photoreceptors other than R8 (Fig. 6A,B), and expression of the EGFR target gene *aos* was strongly reduced in *Hrs* mutant wing discs (Fig. 6C,D), indicating that Hrs is required for EGFR signaling. Loss of *Hrs* did not rescue either photoreceptor differentiation or cell survival in *mop* mutant clones (see Fig. S3G,H in the supplementary material), consistent with a similar function for both proteins in EGFR signaling.

In mammalian cells, EGFR signaling is terminated subsequent to the activity of the ESCRT-I component Tsg101, but before the activity of the ESCRT-III component Vps24 (Bache et al., 2006). Since loss of ESCRT-I and -II complex components activates Notch signaling in *Drosophila*, inhibiting photoreceptor differentiation (Herz et al., 2006; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005), we could not easily evaluate their effects on EGFR signaling in vivo. Instead, we used RNAi to deplete the ESCRT-I complex components Tsg101 and Vps28 from D2F cells treated with Spi. Efficient knockdown was confirmed by RT-PCR and by the enlargement of Hrs-containing endosomes (Fig. 6E; see Fig. S3I-L in the supplementary material). Surprisingly, we found that MAPK phosphorylation was reduced in both cases (Fig. 6F). MAPK phosphorylation was similarly reduced by Cbl depletion (see Fig. S3M in the supplementary material). This suggests that efficient EGFR signaling in *Drosophila* cells requires progression through the endocytic pathway. This model is consistent with the recent finding that human sprouty 2 (SPRY2 – Human Gene Nomenclature Database) antagonizes EGFR signaling by preventing its progression from early to late endosomes (Kim et al., 2007). In S2R+ cells, depleting *sprouty* (*sty*) by RNAi enhanced the cleavage

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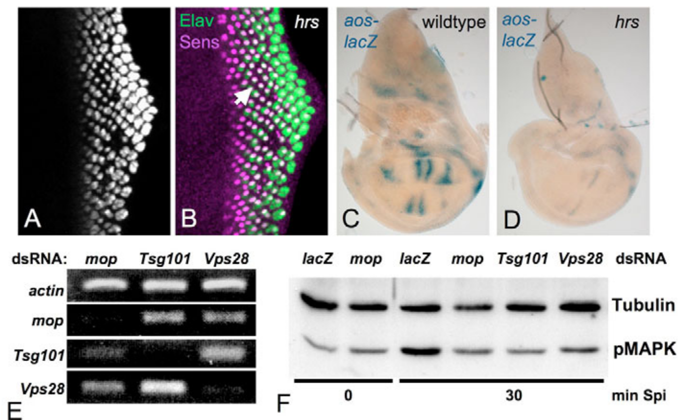

Fig. 6. Progression through endocytosis promotes EGFR signaling. (**A**,**B**) An *Hrs*^{D28}*/Df(2L)Exel6277 Drosophila* eye disc stained with anti-Elav (A, green in B) and anti-Sens (magenta in B). The arrow in B indicates a group of ommatidia containing only R8 photoreceptors. (**C**,**D**) *aos-lacZ* expression in wild-type (C) and *Hrs*^{D28}*/Df(2L)Exel6277* (D) wing discs stained in parallel with X-Gal. R1-7 differentiation and *aos* expression are reduced in *Hrs* mutants. (**E**) Semi-quantitative RT-PCR for *Actin*, *mop*, *Tsg101* and *Vps28* mRNA in D2F cells treated with *mop*, *Tsg101* or *Vps28* dsRNA, demonstrating efficient knockdown in each case. (**F**) Western blot with antibodies to Tubulin and diphospho-MAPK of lysates from D2F cells treated with *lacZ*, *mop*, *Tsg101* or *Vps28* dsRNA and incubated with Spi for 0 or 30 minutes. Depletion of either Mop or the ESCRT-I complex components reduced MAPK phosphorylation.

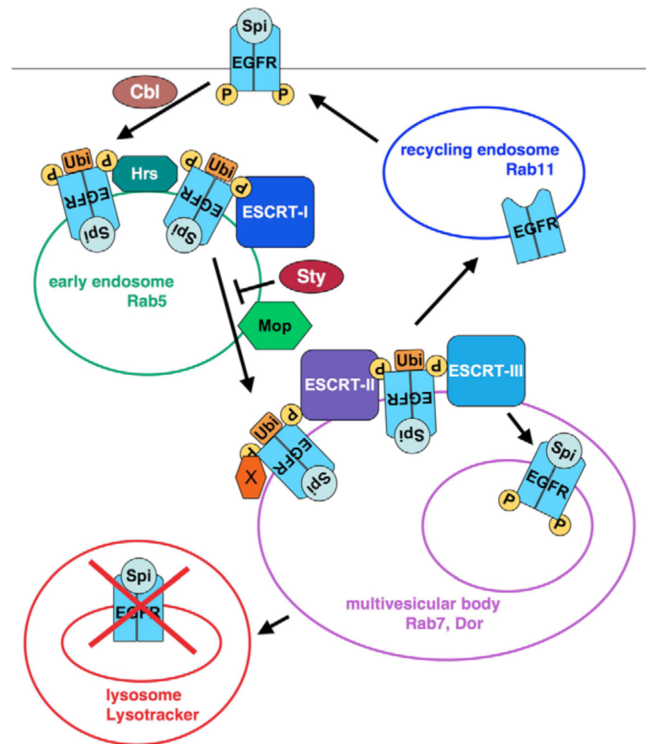

Fig. 7. Model for the effect of Mop on EGFR endocytosis in *Drosophila*. Upon activation of EGFR, ubiquitylation by Cbl induces EGFR internalization through clathrin-coated vesicles. These vesicles fuse with early endosomes and the EGFR is passed from the Hrs complex to the ESCRT-I, ESCRT-II and ESCRT-III complexes as the endosomes are transformed into multivesicular bodies (MVBs). ESCRT-III promotes EGFR deubiquitylation and entry into the internal vesicles of MVBs; fusion of MVBs with lysosomes results in EGFR degradation. Sprouty prevents the EGFR from progressing into late endosomes. We propose that Mop is required for EGFR progression through the endocytic pathway, perhaps through its effect on Hrs. This progression may allow EGFR to encounter crucial downstream components located on late endosomes (X), or to be recycled to the plasma membrane to prolong signaling. Cleavage of the receptor must occur at a stage after the requirement for *mop*.

of EGFRλtop (Fig. 5G), supporting a function for *Drosophila* Sty in blocking EGFR progression through endocytosis. Removal of *sty* restored photoreceptor differentiation to *mop* mutant cells (Fig. 2O,P) and partially rescued MAPK phosphorylation in Mop-depleted cells (see Fig. S3M in the supplementary material), suggesting that Mop might counteract Sty activity.

Mop might affect EGFR trafficking either through a direct interaction or through an indirect effect on the endocytic pathway. We were unable to coimmunoprecipitate Mop with either wild-type or activated EGFR from S2 cells (data not shown), and activated EGFR expressed in vivo showed a vesicular distribution distinct from that of coexpressed Mop (Fig. 4O), suggesting an indirect effect. Nevertheless, Mop does not act indiscriminately on all endocytosed receptors. The Hh target gene *decapentaplegic* (*dpp*) (Heberlein and Moses, 1995) was expressed normally in *mop* mutant clones in the eye disc (see Fig. S5C in the supplementary material), and Ato expression resolved into single R8 cells, indicating normal Notch signaling (Fig. 1B-B″). In the wing disc, *mop* mutant clones likewise showed normal expression of Notch and Hh target genes (see Fig. S5A,B in the supplementary material). Some *mop* mutant clones in the wing disc showed reduced expression of the Wg target gene *sens* (Parker et al., 2002) (see Fig. S5G,H in the supplementary material) and a corresponding loss of the adult wing margin bristles specified by Sens (see Fig. S5I in the supplementary material), although the low-threshold target gene *Distal-less* (*Dll*) (Zecca et al., 1996) was not significantly affected (see Fig. S5J,K in the supplementary material). In these clones, Wg protein accumulated in punctate structures that often colocalized with Hrs (see Fig. S5D-F in the supplementary material), suggesting that Wg and its Frizzled receptors also require Mop activity for normal endocytic progression.

DISCUSSION

We have shown that the Bro1-domain protein Mop is necessary for EGFR signaling in vivo and in cultured cells. Mop is located on endosomes and affects endosome size, promotes cleavage of EGFR and lysosomal entry of its ligand, and is not required in the absence of the Cbl or Sty proteins that regulate endocytic trafficking of EGFR. These data suggest that Mop enhances EGFR signaling by facilitating its progression through the endocytic pathway (Fig. 7). Consistent with this model, Hrs and ESCRT-I subunits also have a positive effect on EGFR signaling.

Mop homologs regulate endocytic sorting

The Bro1 domain of yeast Bro1 is sufficient for localization to late endosomes through its binding to the ESCRT-III subunit Snf7 (Kim et al., 2005), and this domain is present in many proteins involved in endocytosis. Bro1 itself is required for transmembrane proteins to reach the vacuole for degradation; it promotes protein deubiquitylation by recruiting and activating Doa4, a ubiquitin thiolesterase (Luhtala and Odorizzi, 2004; Odorizzi et al., 2003;

DEVELOPMENT

Richter et al., 2007). Since mutations in the E3 ubiquitin ligase gene *Cbl* can rescue *mop* mutant clones, recruiting deubiquitylating enzymes might be one of the functions of Mop. The vertebrate Bro1-domain protein Alix (also known as AIP1 and Pdcd6ip) inhibits EGFR endocytosis by blocking the ubiquitylation of EGFR by Cbl, and by preventing the binding of Ruk (Sh3kbp1), which recruits endophilins, to the EGFR-Cbl complex (Schmidt et al., 2004). However, CG12876, not Mop, is the *Drosophila* ortholog of Alix (Tsuda et al., 2006).

A closer vertebrate homolog of Mop, which has both Bro1 and tyrosine phosphatase domains, has been named HD-PTP in human (Toyooka et al., 2000) and PTP-TD14 in rat (Cao et al., 1998). HD-PTP shares with Alix the ability to bind Snf7 and Tsg101, but does not bind to Ruk (Ichioka et al., 2007). PTP-TD14 was found to suppress cell transformation by Ha-Ras, and required phosphatase activity for this function (Cao et al., 1998). The activity of Mop that we describe here appears distinct in that Mop acts upstream of Ras activation, and we could not demonstrate a requirement for the catalytic cysteine in its predicted phosphatase domain. If Mop does act as a phosphatase, Hrs would be a candidate substrate because tyrosine phosphorylation of Hrs by internalized receptors promotes its degradation (Stern et al., 2007), and Hrs levels appear reduced in *mop* mutant clones.

Endocytosis and receptor signaling

Endocytosis has been proposed to play several different roles in receptor signaling. Most commonly, endocytosis followed by receptor degradation terminates signaling. However, endocytosis can also prolong the duration of signaling (Jullien and Gurdon, 2005) or influence its subcellular location (de Souza et al., 2007; Howe and Mobley, 2005). Receptors may also signal through different downstream pathways localized to specialized endosomal compartments (Di Guglielmo et al., 2003; Miaczynska et al., 2004; Teis et al., 2006).

Genetic studies in *Drosophila* have emphasized the importance of endocytic trafficking for receptor silencing. Mutations in *Hrs*, *Vps25* or *Tsg101* result in the accumulation of multiple receptors on the perimeter membrane of the MVB, leading to enhanced signaling (Herz et al., 2006; Jekely and Rorth, 2003; Jekely et al., 2005; Lloyd et al., 2002; Moberg et al., 2005; Thompson et al., 2005; Vaccari and Bilder, 2005). Depletion of *Hrs* or *Tsg101* in mammalian cells also results in increased EGFR signaling, although the two molecules have distinct effects on MVB morphology (Lu et al., 2003; Razi and Futter, 2006). By contrast, we find that *mop* and *Hrs* mutants exhibit diminished EGFR signaling in vivo, and depletion of *mop*, *Tsg101* or *Vps28* reduces EGFR signaling in S2 cells. Progression through the endocytic pathway may thus be required for maximal EGFR signaling, at least in some contexts.

Several possible mechanisms could explain such a requirement for endocytic progression (Fig. 7). MAPK phosphorylation may be enhanced in the presence of signaling components present on late endosomes (Kim et al., 2007; Teis et al., 2006). Cleavage of the EGFR cytoplasmic domain, which requires Mop activity, might enhance EGFR signaling. The cleaved intracellular domain of ErbB4 has been shown to enter the nucleus and regulate gene expression (Sardi et al., 2006), suggesting the possibility that Mop affects a nuclear function of EGFR in addition to promoting MAPK phosphorylation. Alternatively, the reduction in EGFR signaling in *mop* mutants could be due to a failure to recycle the receptor to the cell surface. Mutations in the yeast Vps class C genes, which are required for trafficking to late endosomes, also prevent the recycling of cargo proteins (Bugnicourt et al., 2004). Recycling is essential for EGFR-induced proliferation of mammalian cells (Tran et al., 2003), and may promote the localized RTK signaling that drives directional cell migration (Jekely and Rorth, 2003).

Specificity of *mop* function

Despite the reduction in EGFR signaling in *mop* mutants, signaling by other receptors such as Notch, Smoothened and Torso is unaffected. This phenotypic specificity could be due to a dedicated function of Mop in the EGFR pathway, or to high sensitivity of EGFR signaling to a general process that requires Mop. Although the Mop-related protein Alix has been found in a complex with EGFR (Schmidt et al., 2004), we could not detect any physical interaction of Mop with EGFR. The function of *mop* is not limited to promoting EGFR signaling; it also promotes trafficking of Wg and expression of the Wg target gene *sens*. In addition, *mop* is required for normal cellularization of the embryo, and its cellularization phenotype is not rescued by removal of *Cbl* (data not shown).

Additional studies will be required to determine whether all endosomes, or only a specific subclass, are affected by *mop*. Interestingly, EGF treatment of mammalian cells induces EGFR trafficking through a specialized class of MVBs (White et al., 2006). Although we do not see significant colocalization of activated EGFR with Mop, EGFR may transiently pass through Mop-containing endosomes before accumulating in another compartment. The wing disc appears less sensitive than the eye disc to the effect of *mop* on EGFR signaling. This might be due to differences in the endogenous levels of Cbl or other mediators of EGFR internalization, or in the strength or duration of signaling necessary to activate target genes, or to the use of a different ligand with distinct effects on receptor trafficking.

Taken together, our results identify a positive role for progress through the endocytic pathway and for the novel molecule Mop in EGFR signaling in *Drosophila*. The importance of upregulation of the trafficking proteins Rab11a, Rab5a and Tsg101 for EGFR signaling in hepatomas and breast cancers (Fukui et al., 2007; Oh et al., 2007; Palmieri et al., 2006) highlights the potential value of specific effectors of EGFR endocytosis as targets for anti-cancer therapies.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/11/1913/DC1

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