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RESEARCH REPORT

Ubpy controls the stability of the ESCRT-0 subunit Hrs in development

Junzheng Zhang[1,2], Juan Du[1,2], Cong Lei[1], Min Liu[1,2] and Alan Jian Zhu[1,2,*]

ABSTRACT

Ubiquitylated developmental membrane signaling proteins are often internalized for endocytic trafficking, through which endosomal sorting complexes required for transport (ESCRT) act sequentially to deliver internalized cargos to lysosomes. The ESCRT function in endocytic sorting is well established; however, it is not fully understood how the sorting machinery itself is regulated. Here, we show that Ubiquitin isopeptidase Y (Ubpy) plays a conserved role *in vivo* in the homeostasis of an essential ESCRT-0 complex component Hrs. We find that, in the absence of *Drosophila Ubpy*, multiple membrane proteins that are essential components of important signaling pathways accumulate in enlarged, aberrant endosomes. We further demonstrate that this phenotype results from endocytic pathway defects. We provide evidence that Ubpy interacts with and deubiquitylates Hrs. In *Ubpy*-null cells, Hrs becomes ubiquitylated and degraded in lysosomes, thus disrupting the integrity of ESCRT sorting machinery. Lastly, we find that signaling proteins are enriched in enlarged endosomes when *Hrs* activity is abolished. Together, our data support a model in which Ubpy plays a dual role in both cargo deubiquitylation and the ESCRT-0 stability during development.

KEY WORDS: Developmental signaling, Endocytic machinery, ESCRT-0, Hrs, Ubpy, *Drosophila*

INTRODUCTION

Many developmental signaling events are initiated through ligand binding to respective receptors at the plasma membrane. Following uptake into endocytic vesicles referred to as early or sorting endosomes, membrane receptors may recycle back to cell surface via tubular recycling endosomes. However, when directed by a ubiquitin (Ub) signal, receptor complexes are captured and sorted towards lysosomal degradation (Henne et al., 2011). The Ub moieties of protein cargos are recognized by ESCRT complexes, and are sorted into invaginating multivesicular bodies (MVBs). Mature MVBs fuse with lysosomes to deliver protein cargos for degradation (Raiborg and Stenmark, 2009). ESCRT-0 is composed of heterodimers of two subunits: Hrs and Stam. Both subunits bind Ub and clathrin, and Hrs additionally has an FYVE zinc-finger domain that binds phosphatidylinositol 3-phosphate. The ability to bind both lipid and Ub allows ESCRT-0 to initiate endosomal sorting (Clague et al., 2012).

Ubpy (or USP8), a USP family deubiquitinase (DUB), participates in sorting of ubiquitylated receptors through its interaction with ESCRT-0 (Mizuno et al., 2005; Row et al., 2006). Most studies of Ubpy focus on endosomal trafficking of growth factor receptor tyrosine kinases (RTKs) in cultured vertebrate cells. However, conflicting data have been reported. In some cases, reduced Ubpy activity results in accumulation of ubiquitylated cargos (Bowers et al., 2006; Mizuno et al., 2006; Row et al., 2006; Alwan and van Leeuwen, 2007). Other studies suggest that Ubpy promotes RTK stability (Mizuno et al., 2005; Niendorf et al., 2007; Berlin et al., 2010). The role of Ubpy in *Drosophila* development is equally controversial. It was suggested that the Hedgehog (Hh) signaling activator Smoothened (Smo) is subject to Ubpy control (Li et al., 2012; Xia et al., 2012). However, Mukai et al. (Mukai et al., 2010) found that Smo abundance is unchanged when *Ubpy* is depleted.

In view of these discrepancies, we examined the *in vivo* consequence of loss of *Ubpy* activity in *Drosophila*. Our results show that, in addition to the reported function on deubiquitylating membrane proteins, Ubpy deubiquitylates Hrs, a key component of ESCRT-0. Altered Hrs ubiquitylation leads to Hrs degradation in lysosomes, thereby regulating subcellular localization of multiple signaling molecules important for *Drosophila* development.

RESULTS AND DISCUSSION

Ubpy is required for localization of multiple signaling proteins in developing *Drosophila* wing

In an *in vivo* RNAi screen targeting *Drosophila* Ub-proteasome system (UPS) genes (Du et al., 2011; Zhang et al., 2012), we found that inhibiting *Ubpy* activity resulted in larval wing disc deformation and adult fly lethality, consistent with an essential role of *Ubpy* in the developing wing (Mukai et al., 2010). Correct patterning of adult wing relies on interplay among several signaling systems, including Hh, Notch (N) and Wingless (Wg) signaling. To explore which pathway(s) is regulated by *Ubpy*, we examined the expression of core components of these pathways in *Ubpy* RNAi-expressing wing discs. Surprisingly, we found that multiple signaling molecules, including Smo and the Hh signaling receptor Patched (Ptc), the N signaling ligand Delta (Dl) and receptor N, and the Wg signaling receptor Frizzled2 (Fz2), were all mislocalized as large puncta in wing epithelial cells (supplementary material Fig. S1A-H). We excluded the possibility that this phenotype was due to RNAi off-target effects as two additional *Ubpy* RNAi targeting distinct regions of *Ubpy* exhibited the same effect (supplementary material Fig. S1B-F,H). Moreover, we generated $Ubpy^{KO}$ (Mukai et al., 2010) somatic clones in wing discs to eliminate *Ubpy* activity completely. Consistent with RNAi results, Ptc, Smo, N, Dl and Fz2 accumulated in puncta in *Ubpy*-null cells (Fig. 1A-L). Similar results were obtained in eye discs, suggesting a general requirement of Ubpy for signaling protein localization (supplementary material Fig. S2A-F).

Our results add another layer of complexity to Ubpy regulation of Smo as other groups observed either no change (Mukai et al., 2010) or reduced Smo expression (Li et al., 2012; Xia et al., 2012) in *Ubpy*

[1]State Key Laboratory of Biomembrane and Membrane Biotechnology, Ministry of Education Key Laboratory of Cell Proliferation and Differentiation, Peking-Tsinghua Center for Life Sciences, College of Life Sciences, Peking University, Beijing 100871, China. [2]Department of Cellular & Molecular Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, OH 44195, USA.

*Author for correspondence (zhua@pku.edu.cn)

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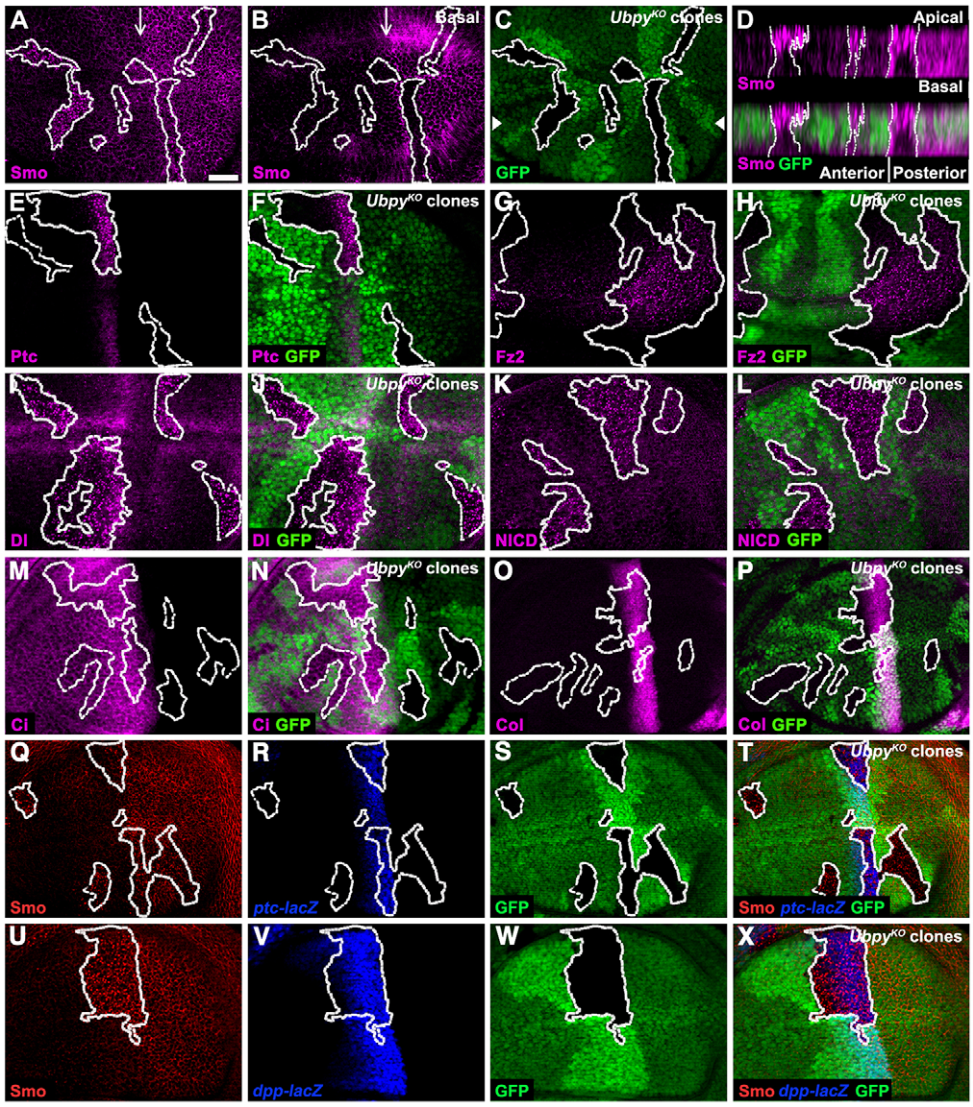

Fig. 1. *Ubpy* regulates subcellular localization of developmental signaling proteins in wing discs. (A-C) Smo accumulation as puncta in $Ubpy^{KO}$ clones (marked by absence of GFP). Note downregulated Smo at basal-most focal plane in the posterior compartment (B). Anterior-posterior border is indicated by arrows. (D) Optical cross-sections along the anterior-posterior axis (as indicated by arrowheads in C) shows Smo accumulation in both anteriorally and posteriorally localized clones. (E-L) Similar localization defect for Ptc (E,F), Fz2 (G,H), Dl (I,J) and N (K,L) in wing discs. (M-X) Ci (M,N) and Col (O,P) expression is unaltered in $Ubpy^{KO}$ clones. Similarly, no obvious defect is observed on *ptc-lacZ* (R) and *dpp-lacZ* activities (V), although mis-localization of Smo is evident (Q,U). Somatic clones are circled by dashed lines. Scale bar: 50 μm.

mutant cells. As wing discs are composed of columnar epithelial cells and Smo subcellular localization is biased towards basolateral domains (Denef et al., 2000), we speculate that images acquired from a single focal plane may not faithfully reflect the distribution of actively trafficking protein cargos. Therefore, we re-examined Smo localization in *Ubpy* RNAi and *Ubpy*-null cells. We found that the reported Smo downregulation (Li et al., 2012; Xia et al., 2012) could only be detected at the basal-most focal plane in the posterior compartment of wing discs (Fig. 1B; supplementary material Fig. S1E). By contrast, in the same cell, Smo aggregates were evident when moving up to apical domains (Fig. 1A; supplementary material Fig. S1B-D); this result is best illustrated in reconstituted optical cross-sections along the *z*-axis (Fig. 1D). More significantly, in the anterior compartment, where Hh signaling is active, the only defect noted was Smo accumulation at apical domains of wing cells (Fig. 1A,D). It is interesting to note that polarized Smo accumulation upon *Ubpy* downregulation was not observed for other membrane proteins examined (supplementary material Fig. S3).

Fly Smo traffics between internal vesicles and plasma membrane. Cell surface localization of Smo is required for Hh signaling activation (Zhu et al., 2003). We next investigated consequences of Smo mis-localization in Hh signaling by monitoring the expression of Hh signaling-responsive genes: Ci and Col (also known as Kn) as well as *ptc-lacZ* and *dpp-lacZ* reporters. Surprisingly, the expression of these markers was not obviously affected by *Ubpy* RNAi in dorsal compartment of wing discs (supplementary material Fig. S1I-L). Note that a slight expansion of *ptc-lacZ* and *dpp-lacZ* expression domains (<15% penetrance, n>50) was observed in cells in which *Ubpy* was massively knocked down (supplementary material Fig. S1M,N). Nevertheless, our result is inconsistent with a previous report that *Ubpy* RNAi downregulates Hh signaling when the same condition was applied (Xia et al., 2012). To address this discrepancy, we generated *Ubpy*-null clones in wing discs. Consistent with our RNAi result, Hh signaling was not altered in $Ubpy^{KO}$ clones (Fig. 1M-X). Thus, accumulated Smo at the apical membrane domains caused by reduced *Ubpy* expression may have limited signaling activity. This might indicate that apical-basal localization of Smo is not essential for its activity. However, we favor another explanation that the lack of effect of mis-localized Smo may result from simultaneous accumulation of Ptc in puncta (Fig. 1E; supplementary material Fig. S1A); Ptc negatively controls Smo localization and activity (Zhu et al., 2003; Torroja et al., 2004). In contrast to the lack of effect of *Ubpy* on Hh signaling, reduced Wg signaling in $Ubpy^{KO}$ clones (supplementary material Fig. S4F; Mukai et al., 2010) and loss of margin bristles caused by *Ubpy* RNAi (Zhang et al., 2012) were observed.

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Developmental signaling proteins are trapped in enlarged endosomal vesicles

Our observation of mis-localized signaling proteins in *Ubpy*^{KO} cells raises an intriguing possibility that endocytic trafficking itself may be affected. To test this hypothesis, we first examined the distribution of early and late endosomes (marked by Rab5 and Rab7, respectively) in *Ubpy*^{KO} cells. Both Rab5-positive early endosomes and Rab7-postive late endosomes were significantly enlarged in *Ubpy*^{KO} cells compared with those in wild-type cells (Fig. 2A-P). Utilizing an FK2 antibody, we found that ubiquitylated proteins were accumulated in these enlarged vesicles (Fig. 2Q-X). The aberrant appearance of these vesicles indicates that Ubpy is required for a key step in the endocytic pathway.

We next used Smo and Dl as examples to examine if accumulated signaling proteins were mis-localized in enlarged vesicles. We found that both Rab5 and Rab7 were present in >80% of Smo- or Dl-containing puncta. This result indicates that sorting of membrane signaling molecules is stalled in enlarged, aberrant vesicles that may have mixed endosomal identities. To explore this possibility further, we co-labeled Smo in *Ubpy*^{KO} clones with another routinely used early endosomal marker, Hrs (Raiborg et al., 2001). To our surprise, Hrs protein was largely undetectable in *Ubpy*^{KO} cells (Fig. 3A-A″). This result is in direct contrast to Rab5 accumulation in enlarged vesicles (Fig. 2), suggesting that Ubpy may directly regulate endocytic sorting at the level of Hrs.

Ubpy protects Hrs from lysosomal degradation

To examine if Ubpy regulates Hrs protein stability, we treated wing discs bearing *Ubpy*^{KO} clones with either lysosomal or proteasomal inhibitors. Blocking lysosomal (E64 and chloroquine; Fig. 3C; supplementary material Fig. S4C-E) but not proteasomal (MG132; Fig. 3B; supplementary material Fig. S4B) activity stabilized Hrs, suggesting that Ubpy protects Hrs from lysosomal degradation. To validate our data obtained from pharmacological inhibition, we utilized well-characterized mutant alleles to disrupt proteasome function or specific steps of endocytic lysosomal trafficking. Disrupting the 20S proteasome core component b6 (Pros26) activity by overexpressing dominant-negative *DTS5* (Belote and Fortier, 2002) failed to rescue *Ubpy* RNAi-induced Hrs degradation (supplementary material Fig. S4H). The *Drosophila* Vps18 homolog Deep orange (Dor) is a member of the class C Vps/HOPS complex that regulates late endosome-to-lysosome transition (Sevrioukov et al., 1999; Sriram et al., 2003). Consistent with a role of Dor in cargo delivery to lysosomes, Hrs was found accumulated in *dor*^{8} mutant wing cells (Fig. 3D). Next, we examined Hrs in *vps22*^{ZZ13} or *vps2*^{PP6} mutant clones, which disrupt ESCRT-II and -III function, respectively

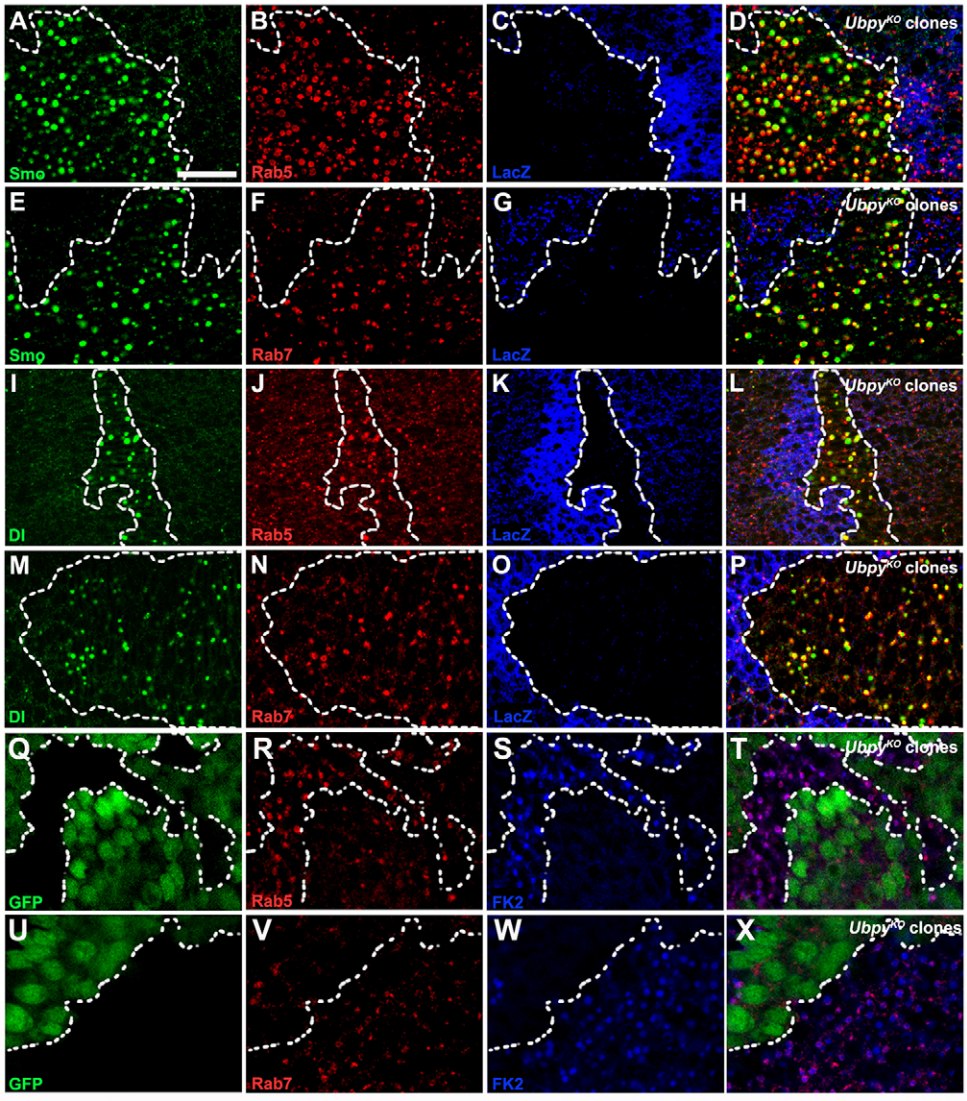

Fig. 2. Signaling proteins are trapped in aberrant vesicles in *Ubpy*^{KO} cells. (A-H) Accumulated Smo colocalizes with the early- and late-endosomal markers Rab5 (A-D) and Rab7 (E-H). Note that both Rab5- and Rab7-positive vesicles are enlarged in *Ubpy*^{KO} cells (marked by absence of *lacZ*). (I-P) Dl is trapped in aberrant vesicles that are positive for both Rab5 (I-L) and Rab7 (M-P). Overall, >80% of Smo- or Dl-bearing vesicles are positive for Rab5 or Rab7 in *Ubpy*^{KO} cells, compared with <15% random colocalization in which one of the two images is rotated [*n*(field of view)=5-7]. (Q-X) In *Ubpy*^{KO} clones (marked by absence of GFP), ubiquitylated cargos are enriched in enlarged vesicles positive for Rab5 (Q-T) or Rab7 (U-X). Somatic clones are circled by dashed lines. Scale bar: 20 μm.

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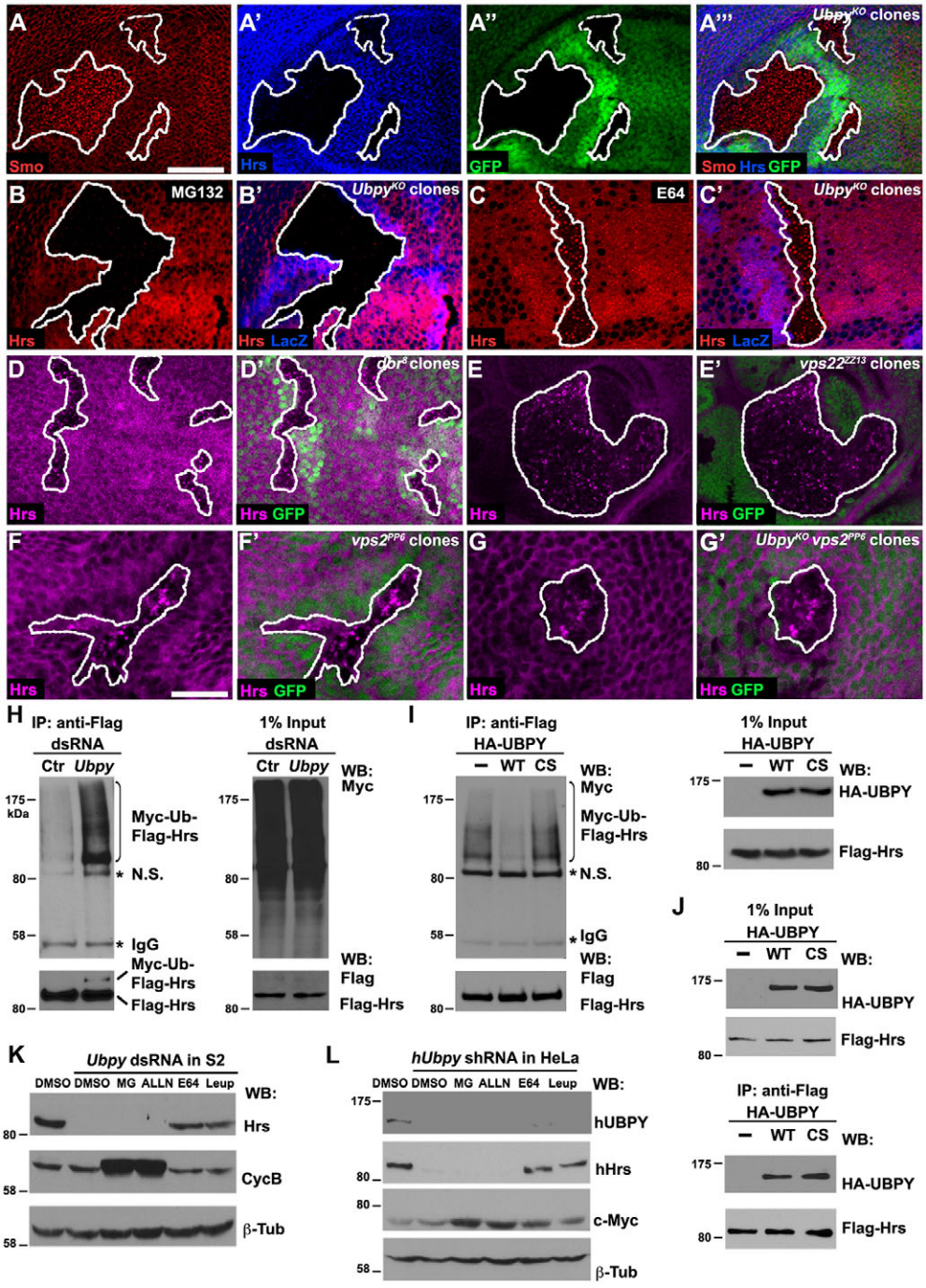

Fig. 3. *Ubpy* protects Hrs from lysosomal degradation. (A-C′) Despite Smo accumulation (A), Hrs (A′) is largely undetectable in $Ubpy^{KO}$ clones, which are marked by lack of GFP (A″) or *lacZ* (B′,C′). Disrupting lysosome function by E64 prevents Hrs degradation (C), whereas inhibiting the UPS function by MG132 (B) has no effect. (D-G′) Hrs accumulation as puncta in dor^{8} (D), $vps22^{ZZ13}$ (E) or $vps2^{PP6}$ (F) clones (marked by lack of GFP). $Ubpy^{KO}$, $vps2^{PP6}$ double mutant clones (G) show the same phenotype as that of $vps2^{PP6}$ single mutant clones (F). Scale bars: 50 μm (A-E′); 20 μm (F-G′). Somatic clones are circled by dashed lines. (H) *Ubpy* RNAi results in enhanced Hrs ubiquitylation in S2 cells. Control blots are shown in supplementary material Fig. S5C. (I,J) HA-tagged wild-type (WT) but not catalytically dead (CS) Ubpy suffices to deubiquitylate Hrs (I). Both forms of Ubpy interact equally with Hrs (J). (K) *Ubpy* dsRNA treatment leads to degradation of endogenous Hrs in S2 cells. This effect is prevented by lysosome (E64 and leupeptin) but not by proteasome inhibitors (MG132 and ALLN). Cyclin B is degraded through proteasomes (Tokumoto et al., 1997), therefore it serves as a positive control for proteasome inhibitors. (L) shRNA knockdown of human UBPY (*hUbpy*) in HeLa cells results in lysosome-mediated degradation of human HRS (hHrs). c-Myc is degraded through proteasomes (Gregory and Hann, 2000), therefore it serves as a positive control for proteasome inhibitors.

(Vaccari et al., 2009). In both cases, Hrs accumulated in aggregates, although the size of aggregates in $vps2^{PP6}$ cells (Fig. 3F) was much larger than that in $vps22^{ZZ13}$ or in dor^{8} cells (Fig. 3D,E). Our genetic data highlight the physiological relevance of regulation of Hrs accumulation by the endocytic lysosomal pathway. To demonstrate a genetic relationship between *Ubpy* and endocytic effectors, $Ubpy^{KO}$ and $vps2^{PP6}$ double mutant clones or cells expressing dsRNAs specific for *Ubpy* and *Vps25* (an ESCRT-II component) were produced in wing discs. Both $vps2^{PP6}$ and *Vps25* RNAi were able to rescue the Hrs degradation that resulted from reduced *Ubpy* activity (Fig. 3G; supplementary material Fig. S4J; compare with Fig. 3A), indicating that *Vps2* and *Vps25* act epistatically to *Ubpy* for Hrs degradation. Taken together, our data demonstrate that Ubpy protects Hrs from lysosomal degradation *in vivo*.

Ubpy removes Ub from its substrates to regulate substrate abundance, localization or activity. The observation that endogenous Hrs co-immunoprecipitated with HA-Ubpy in *Drosophila* S2 cells suggests that Hrs may serve as a Ubpy substrate (supplementary material Fig. S5A). Indeed, Hrs ubiquitylation was increased in *Ubpy* dsRNA-treated cells (Fig. 3H). Conversely, overexpressed wild-type Ubpy was sufficient to reduce the extent of Hrs ubiquitylation, whereas a catalytically dead Ubpy failed to deubiquitylate Hrs (Fig. 3I). Given that both wild-type and mutant Ubpy were expressed at similar levels (Fig. 3I) and displayed a similar degree of Hrs association (Fig. 3J), we conclude that DUB activity of Ubpy is required for the control of Hrs ubiquitylation.

Next, we studied the physiological significance of Ubpy-regulated Hrs ubiquitylation in S2 cells. In the presence of Ubpy, endogenous

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Hrs was stable (supplementary material Fig. S5B). However, Hrs accumulation was greatly reduced when *Ubpy* was depleted by RNAi (Fig. 3K). Interestingly, this *Ubpy* RNAi-mediated Hrs degradation is independent of Hrs association with vesicle membrane or ESCRT-I (supplementary material Fig. S5D). Consistent with *in vivo* results, Hrs was protected from degradation when lysosomal activity was inhibited in S2 cells (Fig. 3K). Previous studies show that disrupted *Ubpy* activity leads to moderate reduction of Hrs expression in vertebrate cells (Row et al., 2006; Niendorf et al., 2007; Berlin et al., 2010). However, the physiological relevance and molecular nature of the Ubpy regulation on Hrs is not known. We found that shRNA knockdown of human UBPY resulted in lysosomal degradation of Hrs in HeLa cells (Fig. 3L), highlighting a conserved function of Ubpy on Hrs stability that may be important for the ESCRT-0 homeostasis on endosomal sorting.

Loss of *Hrs* activity mimics $Ubpy^{KO}$ defects in *Drosophila*

Hrs-mediated ESCRT-0 initiates cargo sorting on endosomes and is required for *Drosophila* development (Lloyd et al., 2002; Seto and Bellen, 2006; Chanut-Delalande et al., 2010). Our biochemical data suggest that Hrs serves as a Ubpy substrate. Thus, reduced Ubpy activity in wing discs may result in defective ESCRT-0 activity and membrane protein cargo sorting. If this were true, we would expect that removing *Hrs* activity would mimic the endosomal defects observed in $Ubpy^{KO}$ cells. Indeed, signaling molecules accumulated in enlarged vesicles in hrs^{D28} clones induced in wing (supplementary material Fig. S6) and eye discs (supplementary material Fig. S2J-O). Similarly, these vesicles were positive for both Rab5 and Rab7 (Fig. 4A-P). Our observation is consistent with a report that signaling molecules are mis-localized in hrs^{D28} egg chamber cells (Jékely and Rørth, 2003).

The canonical role of Ubpy in endocytic sorting is to regulate cargo protein ubiquitylation. Indeed, overexpressed *Ubpy* suffices to modulate cargo ubiquitylation, leading to cargo stabilization, including Smo and Fz2 (supplementary material Fig. S7) (Mukai et al., 2010; Li et al., 2012; Xia et al., 2012). Our genetic and biochemical analyses led us to propose an additional role of Ubpy in Hrs stabilization in order to maintain the ESCRT-0 homeostasis essential for subsequent cargo sorting towards lysosomes (Fig. 4Q). In the absence of Ubpy, ubiquitylated Hrs (Ub-Hrs) may still function to mediate the formation of internal vesicles as incorporation of both Hrs and cargos into enlarged vesicles was observed (Fig. 2; supplementary material Fig. S8H). But because Hrs is not deubiquitylated, incorporated Ub-Hrs is ultimately degraded. Unlike Ub-Hrs, cargos are trapped in enlarged vesicles (Fig. 4R). These seemingly paradoxical fates of Hrs and cargo proteins could be explained when we consider complex roles of Ubpy and Hrs in endosomal sorting. Ubpy deubiquitylation in early endosomes is required for displacement of ESCRT-0 by ESCRT-III, which facilitates cargo sorting into MVBs (Hasdemir et al., 2009;

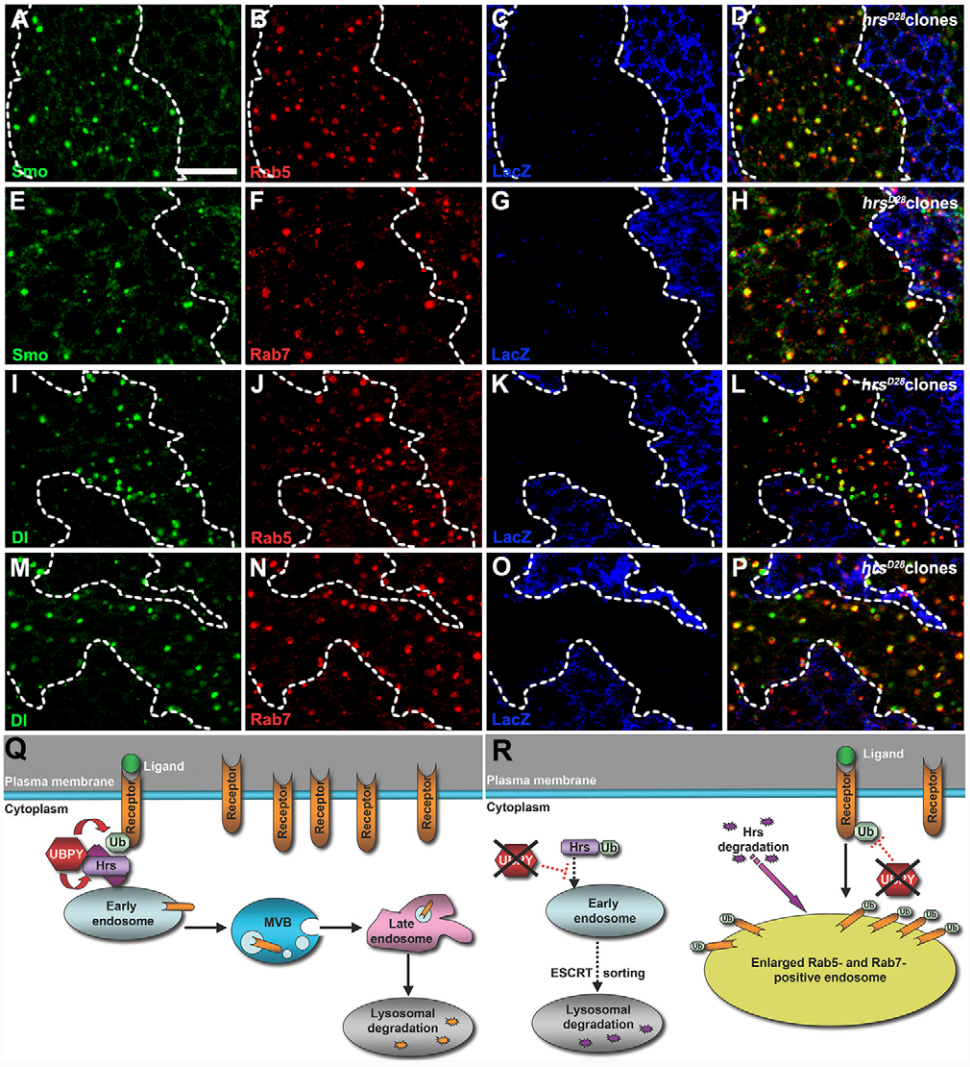

Fig. 4. Signaling proteins are trapped in enlarged endosomes in hrs^{D28} cells. (A-P) Accumulated Smo colocalizes with Rab5 (A-D) and Rab7 (E-H), forming enlarged, aberrant endosomal vesicles in hrs^{D28} clones (marked by absence of *lacZ*). Similarly, Dl colocalizes with Rab5 (I-L) and Rab7 (M-P). Overall, >80% of Smo- or Dl-bearing vesicles are positive for Rab5 or Rab7 in hrs^{D28} cells [*n*(field of view)=5-7]. Somatic clones are circled by dashed lines. Scale bar: 20 µm. (Q,R) Shown is a dual role model of Ubpy functioning in endocytic trafficking. Apart from the canonical role of Ubpy on ubiquitylated cargos in early endosomes, Ubpy may play an addition role in protecting Hrs from ESCRT-mediated lysosomal degradation (Q). In the absence of *Ubpy*, Ub-Hrs may still be capable of mediating endocytic sorting. As the result, incorporated Ub-Hrs is degraded in the lysosome. Upon Hrs depletion, cargo proteins are no longer able to complete endosomeal trafficking for degradation, leading to cargo accumulation in enlarged vesicles with mixed endosomal identity (R).

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Ali et al., 2013). In the absence of Ubpy, Ub-Hrs might be inefficient to sort its cargos into MVBs due to its reduced ability to interact with cargos (Polo et al., 2002; Miller et al., 2004). Furthermore, upon Hrs depletion, endosomal sorting may also be stalled. Thus, cargos may fail to complete endosomal sorting for degradation, resulting in cargo accumulation in enlarged vesicles. At the same time, Ub-Hrs may be recognized by as yet unknown factors that facilitate Hrs degradation in the lysosome. Further experiments are needed to explore these two possibilities.

Hrs degradation upon *Ubpy* depletion seems to have occurred earlier than cargo accumulation in enlarged vesicles (supplementary material Fig. S8A-F), suggesting that these two events might take place sequentially. This interpretation is consistent with aberrant endosomal vesicles associated with reduced Hrs activity (Fig. 4) (Komada and Soriano, 1999; Kanazawa et al., 2003; Hanyaloglu et al., 2005; Lu and Bilder, 2005; Rives et al., 2006; Raiborg et al., 2008; Chanut-Delalande et al., 2010; Li et al., 2012). The *Hrs* and *Ubpy* mutant phenotypes are intriguing in the context of current understanding of sequential ESCRT actions in cargo sorting. Hrs-mediated ESCRT-0 recruits ESCRT-I through interactions with Tsg101 (Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003). However, reduced Tsg101 activity results in a different phenotype; MVB formation is inhibited (Doyotte et al., 2005; Razi and Futter, 2006). We believe that Hrs and Ubpy must play additional roles other than simply recruiting ESCRT-I. This notion is supported by a recent report that ESCRT-0 and Ubpy work together with ESCRT-III to facilitate cargo sorting to MVBs (Ali et al., 2013).

MATERIALS AND METHODS

Fly genetics

ap-Gal4 (Du et al., 2011), *MS1096*-Gal4 (Zhang et al., 2012), *ptc-lacZ*, *dpp-lacZ* (Su et al., 2011), dor^{8} (gift of Helmut Krämer) (Sevrioukov et al., 1999), hrs^{D28} (gift of Hugo Bellen) (Lloyd et al., 2002), $Ubpy^{KO}$ (gift of Satoshi Goto) (Mukai et al., 2010), $vps2^{PP6}$ and $vps22^{ZZ13}$ (gift of David Bilder) (Vaccari et al., 2009) alleles were described previously. Transgenic RNAi flies targeting different regions of *Ubpy* gene were obtained from Dr Satoshi Goto (Mitsubishi-Kagaku Institute of Life Sciences, Machida, Japan) (5798R-1 and 5798R-2) and the Vienna *Drosophila* RNAi Center (VDRC #107623). UAS-*DTS5* (Bloomington #6786), *Hrs* RNAi (TRiP #28026) and *Vps25* RNAi (VDRC #38821) transgenic flies were obtained from Bloomington and VDRC, respectively. *UAS-FLAG-Ubpy* transgenic fly was a gift of Dr Jianhang Jia (Xia et al., 2012).

All fly crosses were maintained at 25°C unless noted otherwise. $Ubpy^{KO}$ and $vps2^{PP6}$ alleles were recombined to chromosome III by homologous recombination. Loss-of-function somatic clones were induced in the wing and eye discs by Flp/FRT-mediated homologous recombination; second-instar larvae from parental crosses were heat-shocked at 37°C for one hour. Specific fly strains and cross conditions as shown in figures are listed in supplementary material Table S1. All phenotypes in wing and eye discs are fully penetrant (n>20) except for those shown in supplementary material Fig. S1M,N.

Immunofluorescence staining

Wing discs dissected from third-instar larvae were fixed in 4% paraformaldehyde and labeled overnight at 4°C with the following primary antibodies: rabbit anti-Ase (1:400; gift of Cheng-Yu Lee, University of Michigan, MI, USA), rat anti-Ci [1:50; 2A1; Developmental Studies Hybridoma Bank (DSHB)], mouse anti-Col (1:100; gift of Alain Vincent, Centre de Biologie du Developpement, CNRS, Toulouse, France), mouse anti-Dl (1:200; C594.9B; DSHB), mouse anti-Fz2 (1:20; 12A7; DSHB), guinea pig anti-Hrs (1:200; gift of Hugo Bellen, Baylor College of Medicine, HHMI, TX, USA), mouse anti-NICD (1:200; C17.9C6; DSHB), mouse anti-Ptc (1:200; Apa1; DSHB), rabbit anti-Rab5 (1:100; gift of Marcos González-Gaitán, University of Geneva, Switzerland), rabbit anti-Rab7 (1:2000; gift of Akira Nakamura, Institute of Molecular Embryology and Genetics, Kumamoto, Japan), mouse anti-Smo (1:20; 20C6; DSHB), chicken anti β-galactosidase (1:200; ICL Lab), rabbit anti β-galactosidase (1:4000; Cappel) and mouse anti-ubiquitylated proteins (1:1000; FK2; Enzo). Alexa Fluor-conjugated secondary antibodies (1:400; Invitrogen) were used. In some experiments, third-instar larvae were dissected and incubated at 25°C for 4 hours in complete *Drosophila* clone-8 cell medium [Shields and Sang M3 insect medium (Sigma) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Invitrogen), 5 mg/ml insulin (Sigma) and 2.5% fly extract, 100 U/ml penicillin and 100 mg/ml streptomycin] supplemented with either lysosomal [E64 (50 μM; Sigma); chloroquine (10 mg/ml; MP Biomedicals)] or proteasomal [MG132 (50 μM; Sigma)] inhibitors before fixation. Fluorescence images were acquired with a Zeiss Axio Imager Z1 microscope equipped with an ApoTome or a Leica SP5 confocal microscope. The figures were assembled in Adobe Photoshop CS5. Minor image adjustments (brightness and/or contrast) were performed in AxioVision 4.8.1 or Adobe Photoshop.

Cell culture, transfection and RNAi treatment

Drosophila Schneider S2 cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 25°C. DNA transfection was carried out using a standard calcium phosphate protocol. *FLAG-Hrs* plasmid was generated by fusing an FLAG tag at the N-terminus of the full-length fly *Hrs* cDNA and then cloned into a pUAST vector. Hrs mutants lacking the FYVE motif and PSAP domain, respectively, were generated by PCR. Wild-type (WT) and catalytically dead (CS) *HA-Ubpy* plasmids were gifts of Dr Jianhang Jia (Xia et al., 2012). *Myc-Ub* plasmid was provided by Dr Shunsuke Ishii (Dai et al., 2003).

dsRNA was generated using the MEGAscript High Yield Transcription Kit (Ambion) according to the manufacturer's instructions. A DNA template targeting *Ubpy* (encoding amino acids 333-435) was generated by PCR and used for dsRNA synthesis. dsRNA targeting yeast *gal80* coding sequence was used as a negative control (Su et al., 2011). For RNAi knockdown, S2 cells were cultured in full medium containing 40 nM indicated dsRNA for 4 days. dsRNA-treated cells were then split and incubated with fresh dsRNA for additional 4 days before harvesting for further experiments.

HeLa cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C. Lipofectamine 2000 (Invitrogen) was used for transfection. RNAi knockdown of human UBPY was achieved by transfecting a shRNA plasmid (TRCN0000007436, Sigma) that has been shown to specifically target *UBPY* in HeLa cells.

In both S2 and HeLa cells, MG132 (50 μM; Sigma) and ALLN (50 μM; Sigma) were used to inhibit the proteasome activity, and E64 (50 μM; Sigma) and leupeptin (50 μM; Sigma) were used to inhibit lysosome function.

Immunoblotting, immunoprecipitation and ubiquitylation assays

S2 and HeLa cells were lysed in NP-40 buffer (1% NP-40, 150 mM NaCl and 50 mM Tris-HCl, pH 8) supplemented with protease inhibitor cocktail (Roche). In some experiments, S2 cells were treated with 50 μg/ml cycloheximide to prevent nascent protein synthesis for up to 8 hours before lysis. Protein concentration of cell lysate was quantified using a BCA Protein Assay Kit (Thermo). Western blot analyses were carried out using standard protocols. The following antibodies were used for immunoblotting: mouse anti-β-Tubulin (1:6000; Covance), mouse anti-cyclin B (1:50; F2F4; DSHB), mouse anti- human HRS (1:1000; A-5; Enzo), guinea pig anti-Hrs (1:2000; gift of Hugo Bellen), rabbit anti-human UBPY (1:1000; #8728; Cell Signaling), rabbit anti-FLAG tag (1:2000; #2368; Cell Signaling), rabbit anti-HA tag (1:1000; Y-11; Santa Cruz), mouse anti-Myc tag (1:2000; 9B11; Cell Signaling) and rabbit anti-endogenous c-Myc (1:1000; D84C12; Cell Signaling). Immunoprecipitation was performed using agarose anti-HA (MB-0734, Vector Laboratories) according to the manufacturer's instructions. Blots presented in all figures are representatives of at least three independent experiments.

Ubiquitylation assays were carried out with hot lysis-extracted protein lysates based on the protocol described previously (Row et al., 2006). Briefly, S2 cells transfected with *FLAG-Hrs* and *Myc-Ub* were treated with 50 μM MG132 and 50 μM E64 for 6 hours before harvesting. Cells were

hot-lysed in 100 µl of denaturing buffer (1% SDS, 50 mM Tris, pH 7.5, 0.5 mM EDTA) by boiling for five minutes at 100°C. Lysates were then diluted 1:10 with NP-40 lysis buffer and subjected to immunoprecipitation using anti-Flag M2 affinity gel (Sigma).

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Competing interests
The authors declare no competing financial interests.

Author contributions
J.Z. and A.J.Z. designed experiments; J.Z., J.D., C.L. and M.L. performed experiments; J.Z. and A.J.Z. analyzed the data and wrote the manuscript.

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References
Ali, N., Zhang, L., Taylor, S., Mironov, A., Urbé, S. and Woodman, P. (2013). Recruitment of UBPY and ESCRT exchange drive HD-PTP-dependent sorting of EGFR to the MVB. *Curr. Biol.* **23**, 453-461.
Alwan, H. A. and van Leeuwen, J. E. (2007). UBPY-mediated epidermal growth factor receptor (EGFR) de-ubiquitination promotes EGFR degradation. *J. Biol. Chem.* **282**, 1658-1669.
Bache, K. G., Brech, A., Mehlum, A. and Stenmark, H. (2003). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. *J. Cell Biol.* **162**, 435-442.
Belote, J. M. and Fortier, E. (2002). Targeted expression of dominant negative proteasome mutants in Drosophila melanogaster. *Genesis* **34**, 80-82.
Berlin, I., Higginbotham, K. M., Dise, R. S., Sierra, M. I. and Nash, P. D. (2010). The deubiquitinating enzyme USP8 promotes trafficking and degradation of the chemokine receptor 4 at the sorting endosome. *J. Biol. Chem.* **285**, 37895-37908.
Bowers, K., Piper, S. C., Edeling, M. A., Gray, S. R., Owen, D. J., Lehner, P. J. and Luzio, J. P. (2006). Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII. *J. Biol. Chem.* **281**, 5094-5105.
Chanut-Delalande, H., Jung, A. C., Baer, M. M., Lin, L., Payre, F. and Affolter, M. (2010). The Hrs/Stam complex acts as a positive and negative regulator of RTK signaling during Drosophila development. *PLoS ONE* **5**, e10245.
Clague, M. J., Liu, H. and Urbé, S. (2012). Governance of endocytic trafficking and signaling by reversible ubiquitylation. *Dev. Cell* **23**, 457-467.
Dai, P., Akimaru, H. and Ishii, S. (2003). A hedgehog-responsive region in the Drosophila wing disc is defined by debra-mediated ubiquitination and lysosomal degradation of Ci. *Dev. Cell* **4**, 917-928.
Denef, N., Neubüser, D., Perez, L. and Cohen, S. M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. *Cell* **102**, 521-531.
Doyotte, A., Russell, M. R., Hopkins, C. R. and Woodman, P. G. (2005). Depletion of TSG101 forms a mammalian "Class E" compartment: a multicisternal early endosome with multiple sorting defects. *J. Cell Sci.* **118**, 3003-3017.
Du, J., Zhang, J., Su, Y., Liu, M., Ospina, J. K., Yang, S. and Zhu, A. J. (2011). In vivo RNAi screen reveals neddylation genes as novel regulators of Hedgehog signaling. *PLoS ONE* **6**, e24168.
Gregory, M. A. and Hann, S. R. (2000). c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol. Cell. Biol.* **20**, 2423-2435.
Hanyaloglu, A. C., McCullagh, E. and von Zastrow, M. (2005). Essential role of Hrs in a recycling mechanism mediating functional resensitization of cell signaling. *EMBO J.* **24**, 2265-2283.
Hasdemir, B., Murphy, J. E., Cottrell, G. S. and Bunnett, N. W. (2009). Endosomal deubiquitinating enzymes control ubiquitination and down-regulation of protease-activated receptor 2. *J. Biol. Chem.* **284**, 28453-28466.
Henne, W. M., Buchkovich, N. J. and Emr, S. D. (2011). The ESCRT pathway. *Dev. Cell* **21**, 77-91.
Jékely, G. and Rørth, P. (2003). Hrs mediates downregulation of multiple signalling receptors in Drosophila. *EMBO Rep.* **4**, 1163-1168.
Kanazawa, C., Morita, E., Yamada, M., Ishii, N., Miura, S., Asao, H., Yoshimori, T. and Sugamura, K. (2003). Effects of deficiencies of STAMs and Hrs, mammalian class E Vps proteins, on receptor downregulation. *Biochem. Biophys. Res. Commun.* **309**, 848-856.
Katzmann, D. J., Stefan, C. J., Babst, M. and Emr, S. D. (2003). Vps27 recruits ESCRT machinery to endosomes during MVB sorting. *J. Cell Biol.* **162**, 413-423.
Komada, M. and Soriano, P. (1999). Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. *Genes Dev.* **13**, 1475-1485.
Li, S., Chen, Y., Shi, Q., Yue, T., Wang, B. and Jiang, J. (2012). Hedgehog-regulated ubiquitination controls smoothened trafficking and cell surface expression in Drosophila. *PLoS Biol.* **10**, e1001239.
Lloyd, T. E., Atkinson, R., Wu, M. N., Zhou, Y., Pennetta, G. and Bellen, H. J. (2002). Hrs regulates endosome membrane invagination and tyrosine kinase receptor signaling in Drosophila. *Cell* **108**, 261-269.
Lu, H. and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in Drosophila. *Nat. Cell Biol.* **7**, 1232-1239.
Lu, Q., Hope, L. W., Brasch, M., Reinhard, C. and Cohen, S. N. (2003). TSG101 interaction with HRS mediates endosomal trafficking and receptor down-regulation. *Proc. Natl. Acad. Sci. USA* **100**, 7626-7631.
Miller, S. L. H., Malotky, E. and O'Bryan, J. P. (2004). Analysis of the role of ubiquitin-interacting motifs in ubiquitin binding and ubiquitylation. *J. Biol. Chem.* **279**, 33528-33537.
Mizuno, E., Iura, T., Mukai, A., Yoshimori, T., Kitamura, N. and Komada, M. (2005). Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes. *Mol. Biol. Cell* **16**, 5163-5174.
Mizuno, E., Kobayashi, K., Yamamoto, A., Kitamura, N. and Komada, M. (2006). A deubiquitinating enzyme UBPY regulates the level of protein ubiquitination on endosomes. *Traffic* **7**, 1017-1031.
Mukai, A., Yamamoto-Hino, M., Awano, W., Watanabe, W., Komada, M. and Goto, S. (2010). Balanced ubiquitylation and deubiquitylation of Frizzled regulate cellular responsiveness to Wg/Wnt. *EMBO J.* **29**, 2114-2125.
Niendorf, S., Oksche, A., Kisser, A., Löhler, J., Prinz, M., Schorle, H., Feller, S., Lewitzky, M., Horak, I. and Knobeloch, K. P. (2007). Essential role of ubiquitin-specific protease 8 for receptor tyrosine kinase stability and endocytic trafficking in vivo. *Mol. Cell. Biol.* **27**, 5029-5039.
Polo, S., Sigismund, S., Faretta, M., Guidi, M., Capua, M. R., Bossi, G., Chen, H., De Camilli, P. and Di Fiore, P. P. (2002). A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. *Nature* **416**, 451-455.
Raiborg, C. and Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458**, 445-452.
Raiborg, C., Bremnes, B., Mehlum, A., Gillooly, D. J., D'Arrigo, A., Stang, E. and Stenmark, H. (2001). FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes. *J. Cell Sci.* **114**, 2255-2263.
Raiborg, C., Malerød, L., Pedersen, N. M. and Stenmark, H. (2008). Differential functions of Hrs and ESCRT proteins in endocytic membrane trafficking. *Exp. Cell Res.* **314**, 801-813.
Razi, M. and Futter, C. E. (2006). Distinct roles for Tsg101 and Hrs in multivesicular body formation and inward vesiculation. *Mol. Biol. Cell* **17**, 3469-3483.
Rives, A. F., Rochlin, K. M., Wehrli, M., Schwartz, S. L. and DiNardo, S. (2006). Endocytic trafficking of Wingless and its receptors, Arrow and DFrizzled-2, in the Drosophila wing. *Dev. Biol.* **293**, 268-283.
Row, P. E., Prior, I. A., McCullough, J., Clague, M. J. and Urbé, S. (2006). The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation. *J. Biol. Chem.* **281**, 12618-12624.
Seto, E. S. and Bellen, H. J. (2006). Internalization is required for proper Wingless signaling in Drosophila melanogaster. *J. Cell Biol.* **173**, 95-106.
Sevrioukov, E. A., He, J. P., Moghrabi, N., Sunio, A. and Krämer, H. (1999). A role for the deep orange and carnation eye color genes in lysosomal delivery in Drosophila. *Mol. Cell* **4**, 479-486.
Sriram, V., Krishnan, K. S. and Mayor, S. (2003). deep-orange and carnation define distinct stages in late endosomal biogenesis in Drosophila melanogaster. *J. Cell Biol.* **161**, 593-607.
Su, Y., Ospina, J. K., Zhang, J., Michelson, A. P., Schoen, A. M. and Zhu, A. J. (2011). Sequential phosphorylation of smoothened transduces graded hedgehog signaling. *Sci. Signal.* **4**, ra43.
Tokumoto, T., Yamashita, M., Tokumoto, M., Katsu, Y., Horiguchi, R., Kajiura, H. and Nagahama, Y. (1997). Initiation of cyclin B degradation by the 26S proteasome upon egg activation. *J. Cell Biol.* **138**, 1313-1322.
Torroja, C., Gorfinkiel, N. and Guerrero, I. (2004). Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. *Development* **131**, 2395-2408.
Vaccari, T., Rusten, T. E., Menut, L., Nezis, I. P., Brech, A., Stenmark, H. and Bilder, D. (2009). Comparative analysis of ESCRT-I, ESCRT-II and ESCRT-III function in Drosophila by efficient isolation of ESCRT mutants. *J. Cell Sci.* **122**, 2413-2423.
Xia, R., Jia, H., Fan, J., Liu, Y. and Jia, J. (2012). USP8 promotes smoothened signaling by preventing its ubiquitination and changing its subcellular localization. *PLoS Biol.* **10**, e1001238.
Zhang, J., Liu, M., Su, Y., Du, J. and Zhu, A. J. (2012). A targeted in vivo RNAi screen reveals deubiquitinases as new regulators of Notch signaling. *G3 (Bethesda)* **2**, 1563-1575.
Zhu, A. J., Zheng, L., Suyama, K. and Scott, M. P. (2003). Altered localization of Drosophila Smoothened protein activates Hedgehog signal transduction. *Genes Dev.* **17**, 1240-1252.