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

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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 ubiquitins 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 (Fzd2), 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\textsuperscript{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
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 (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 membrane domains (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 UbpyKO 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 UbpyKO clones (supplementary material Fig. S4F; Mukai et al., 2010) and loss of margin bristles caused by Ubpy RNAi (Zhang et al., 2012) were observed.
Developmental signaling proteins are trapped in enlarged endosomal vesicles

Our observation of mis-localized signaling proteins in UbpyKO 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 UbpyKO cells. Both Rab5-positive early endosomes and Rab7-positive late endosomes were significantly enlarged in UbpyKO 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 DI 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 DI-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 UbpyKO clones with another routinely used early endosomal marker, Hrs (Raiborg et al., 2001). To our surprise, Hrs protein was largely undetectable in UbpyKO 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 UbpyKO 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) 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; Sirram et al., 2003). Consistent with a role of Dor in cargo delivery to lysosomes, Hrs was found accumulated in dor8 mutant wing cells (Fig. 3D). Next, we examined Hrs in vps22ZZ13 or vps2PP6 mutant clones, which disrupt ESCRT-II and -III function, respectively.
(Vaccari et al., 2009). In both cases, Hrs accumulated in aggregates, although the size of aggregates in vps2\textsuperscript{PP6} cells (Fig. 3F) was much larger than that in vps2\textsuperscript{PP6} or dor\textsuperscript{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\textsuperscript{KO} and vps2\textsuperscript{PP6} double mutant clones or cells expressing dsRNAs specific for Ubpy and Vps25 (an ESCRT-II component) were produced in wing discs. Both vps2\textsuperscript{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\textsuperscript{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 Hrs were degraded more efficiently than in control cells (Fig. 3K). Ubpy\textsuperscript{dsRNA} treatment led 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.

Fig. 3. Ubpy protects Hrs from lysosomal degradation. (A–C) Despite Smo accumulation (A), Hrs (A’) is largely undetectable in Ubpy\textsuperscript{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\textsuperscript{8} (D), vps2\textsuperscript{PP6} (E) or vps2\textsuperscript{PP6} (F) clones (marked by lack of GFP). Ubpy\textsuperscript{KO}, vps2\textsuperscript{PP6} double mutant clones (G) show the same phenotype as that of vps2\textsuperscript{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. SSC. (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.
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 UbpYKO 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 UbpYKO cells. Indeed, signaling molecules accumulated in enlarged vesicles in hrsD28 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 hrsD28 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;...
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 genotypes**

*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*Δ28 (gift of Hugo Bellen) (Lloyd et al., 2002), *UbpyKO* (gift of Satoshi Goto) (Mukai et al., 2010), *vps2PP6* and *vps2Z2ZI* (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) (5799R-1 and 5799R-2) and the Vienna Drosophila RNAi Center (VDRC #107623). UAS-DSY5 (Bloomington #6786), *Hrs* RNAi (TRIP #28026) and *Vps25* RNAi (VDRC #38821) transgenic flies were obtained from Bloomington and VDRC, respectively.

**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 Jianhong Jia (Xia et al., 2012). Mhc-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 *gab1* coding sequence was obtained from 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 lysosomal 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; F242; 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-endoenogenous c-Myc (1:1000; D841C2; 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 with 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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Supplementary information

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