Endosomal sorting is the entry point into the degradative fate and it involves sorting of ubiquitylated cargoes on the limiting membrane of endosomes and the formation of Multi Vesicular Endosomes (MVEs). Endosomal sorting and MVE biogenesis are controlled by Endosomal Sorting Required for Transport (ESCRT) proteins. Four multi-subunit ESCRT complexes (ESCRT-I, -II, -III, -IV) act in a sequential fashion to deliver cargoes into the internal luminal vesicles (ILVs) of the nascent MVE [3–5]. The process is thought to start when the ESCRT-0 components Hrs and Stam, acting as an heterodimer, clusters ubiquitylated cargoes in flat clathrin-coated domains of the endosomal membrane. ESCRT-0 then is thought to recruit the ESCRT-I complex and subsequent action of ESCRT-II and -III complexes leads to de-ubiquitylation of cargoes and their sequestration in forming ILVs [6–9]. The full extent of cargoes subjected to endosomal sorting, and how sorting affects signaling modulation precisely is largely unknown.

Mutants for ESCRT components in metazoan animals, such as Drosophila melanogaster, have been recently providing a fascinating initial glimpse in the importance of endosomal degradation for signaling regulation during development [10]. In fact, in addition to showing failure to degrade a number of transmembrane signaling receptors, they show ectopic activity of multiple signaling pathways, including Notch, JAK/STAT and others [11–16]. In addition, epithelial tissue mutant for a large number of ESCRT
Figure 1. *Hrs, Stam* or *Hrs, Stam* double mutant tissue do not display altered tissue architecture. (A–H) Epithelial morphology of mosaic FE cells (A–D) and eye discs (E–H) revealed by phalloidin staining to detect F-actin. Follicle cells of 5–7 stage egg chambers homozygous for the
genes display altered apico-basal polarity and unrestrained proliferation leading to formation of tumor-like masses, indicating that endosomal sorting, possibly by regulating signal transduction play a major role in tumor suppression [17,18]. Loss of tumor suppression in Drosophila ESCRT mutants requires ectopic activity of Notch, JAK/STAT, and dpp and JNK signaling, as down-modulation of these pathways in ESCRT mutant rescues the overproliferation or the loss of polarity, or both. For instance, ESCRT mutant cells display [and rely on] ectopic, ligand-independent activation of Notch signaling for cell-autonomous proliferation and on ectopic JAK/STAT signaling activation for cell-autonomous non cell-autonomous proliferation [11]. Such dramatic increase of proliferative signaling alters cell cycle regulation and is counteracted by JNK- and Hippo-mediated mediated activation of apoptosis [14,16]. Thus, while the proliferative defects of ESCRT mutants are well documented, how apico-basal polarity is compromised is still obscure. Despite this, consistent with conservation in the involvement of ESCRTs in tumor suppression, a number of ESCRT-I, -II, -III components have been found mis-expressed in various cancers (see for review [19]).

Unexpectedly, while all the Drosophila ESCRT-I, -II, -III genes analyzed so far behave as tumor suppressors and prevent ectopic ligand independent Notch activation, Drosophila Hrs, which encodes for one of the two obligate ESCRT-0 components, is required for endosomal sorting, and signaling attenuation by RTKs, but it appears dispensable for tumor suppression. In addition, in a Hrs mutant, Notch fails to be degraded but it is otherwise normally activated [20–22]. It has been recently reported that mutants in Stam, which encodes for the Hrs partner in ESCRT-0, and Stam Hrs double mutants affect endosomal sorting, MVE biogenesis and alter RTK signaling [23,24]. However, it is not clear whether Stam or Hrs Stam double mutants display loss of tumor suppression or altered Notch trafficking and signaling [23]. Thus, we decided to analyze epithelial tissues that lack function of Stam or both Hrs and Stam during Drosophila development.

Here we show that differently from ESCRT-I, -II, -III mutants, Stam or Hrs, Stam double mutants do not present loss of tumor suppression or ectopically active Notch signaling. However, similarly to single Hrs mutants and other ESCRT mutants, Stam or Hrs, Stam double mutants display endosomal accumulation of ubiquitinated cargos, including Notch and the JAK/STAT receptor Domeless. Unexpectedly, our data indicate that ESCRT-0 is dispensable for tumor suppression and ectopic Notch signaling activation, and shed light on the mechanism of ESCRT-mediated tumor suppression and of endosomal Notch activation.

**Materials and Methods**

**Fly Strains and Genetics**

Drosophila lines referred to in the text are Hrs<sup>D28</sup> [20], Stam<sup>u22896</sup> [24], and the double mutant Hrs<sup>D28</sup> Stam<sup>u22896</sup> (Bloomington Drosophila Stock Center (BDSC) #3914, #41804 and #41806, respectively). Predominantly mutant eye and wing discs (referred to in the text as mutant discs) were generated with the eyeFLP mosaic system as described previously [26]. Mutant FE cell clones were generated by using the heat shock-mosaic system [27] and the GR1 system [28]. For most of the mosaic experiments, female flies were heat-shocked at 37°C for 1 h two times a day for 2 days and then incubated at 25°C for 4 days before dissection. Detailed genotypes are available upon request.

The Hrs, Stam recombinants devoid of l(2)gl lesions were generated via standard genetic procedures. After we made sure that both the Hrs<sup>D28</sup> and Stam<sup>u22896</sup> single mutants did not contain l(2)gl lesions by complementation assay with the null allele l(2)gl<sup>4</sup>, Hrs<sup>D28</sup> males were crossed with Stam<sup>u22896</sup> males to generate recombinogenic F1 females. These were then crossed to a balancer stock and the F2 male progeny was stocked and crossed back to Hrs and Stam mutants and relative deficiencies (Hrs deficiency: BDSC #9543; Stam deficiency BDSC #7821). Males that failed complementation with both loci but complemented l(2)gl<sup>4</sup> or a l(2)gl<sup>4</sup> deficiency (BDSC #3634) were kept as independent recombinant fly lines.

**Immunostainings and Confocal Microscopy**

Ovaries and discs were dissected in PBS, fixed in 4% PFA for 20 minutes at room temperature and then rinsed three times in phosphate buffered saline with 0.1% Triton X-100. To increase permeabilization of the antibody in the tissue, ovaries have been treated for 10 min with 1% triton X-100. Before incubation with primary antibody ovaries and discs have been incubated with a blocking solution composed of 5% BSA in PBS-Triton 0.1%. Primary antibodies were used for immunostaining against the following antigens: Hnt, Cut, Notch ECD, Notch ICD, all from Developmental Studies Hybridoma Bank- DSHB; Dome (A gift from Stephanie Noselli). Avl (Lu and Bilder, 2003); Ubiquitin FK2 (Biomol); activated Caspase-3 (Signal Transduction Technologies). Secondary antibodies conjugated to Alexa-488, Alex-568 were used (Molecular Probes). Phallodin-TRITC from sigma was used to mark F-actin while DAPI (4'6-diamino-2-phenylindole) to stain the nuclei. The images were obtained using a Zeiss LSM 510-Meta confocal microscope or an a1 TCS microscope (Leica). Images were edited with Adobe Photoshop CS and were assembled with Adobe Illustrator.

**Transmission Electron Microscopy**

Eye discs WT or mutant for Stam, Hrs, Stam l(2)gl<sup>4</sup> or Vp<sup>25</sup> were fixed in 2.5% glutaraldehyde diluted in 0.1 M sodium cacodylate buffer for 3 hours at room temperature. Eye discs were post-fixed in 1% osmium tetroxide (Electron Microscopy Science, Hatfield, PA, USA) for 2 hours at room temperature and subsequently in 1% uranyl acetate (Electron microscopy science) for hour. Samples were dehydrated through a graded ethanol series and next in propylene oxide before embedding in epoxy resin (PolyBed, Polysciences, Warrington, PA, USA) overnight at 42°C and then 2 days at 60°C. Searching for the eye disc epithelium was performed on semi-thin sections (500 nm) stained with toluidine blue. Ultrathin sections of 50 nm were then cut and stained with 5% uranyl acetate and lead citrate. Representative TEM micrographs of each sample were taken with Tecnai 12-G2.
Figure 2. ESCRT-0 mutations lead to accumulation of ubiquitylated cargoes, as well as of Notch and Dome in endosomes. (A–F) High magnification of a region of mosaic eye imaginal discs (A–D), or of FE (E–F) shows accumulation of ubiquitylated cargoes in mutant cells (GFP-ESCRT-0 and Tumorigenesis PLOS ONE | www.plosone.org 4 April 2014 | Volume 9 | Issue 4 | e93987
was performed in a subset of samples. Time points. Localization of the anti-Notch EDC antibodies was at the 0 min time point or after 60 min or 300 min for the different cultured for 20 and 60 min, respectively, in presence of anti-Notch and after dissection the medium was replaced. Imaginal discs were fixed immediately for medium changes the organs were fixed immediately for living imaginal discs. In WT tissue, after labeling (0 hrs), Notch is present mostly at the apical surface of the cell. After a 5-hour chase (5 hrs) Notch is completely degraded in WT but still present in endosomes in Stam mutant discs, indicating that Notch is internalized but it is not degraded.

doi:10.1371/journal.pone.0093987.g002

### Notch-trafficking Assay

Wild-type or eyFLP/+; FRT40A Stam2L2896/FRT40A P(mini-w, cl), eyFLP/+; FRT40A Hrs2D8 Stam2L2896/; l(2)gl FRT40A P(mini-w, cl) eye discs were dissected in Schneider’s Drosophila medium and after dissection the medium was replaced. Imaginal discs were cultured for 20 and 60 min, respectively, in presence of anti-Notch ECD antibody that recognizes the extracellular portion of Notch. Following medium changes the organs were fixed immediately for 0 min time point or after 60 min or 300 min for the different time points. Localization of the anti-Notch EDC antibodies was revealed using secondary antibody, and co-staining with anti-Avl was performed in a subset of samples.

### RT- PCR

Total RNA from wing imaginal discs (40 discs per sample) was extracted using TRIZOL Reagent (Invitrogen) and RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Concentration and purity was determined by measuring optical density at 260 and 280 nm using a Nanodrop spectrophotometer. 1 μg of total RNA was reverse transcribed using a SuperScript VILO cDNA Synthesis kit (Invitrogen) according to the manufacturer’s protocol. 5 ng of cDNA was amplified (in triplicate) in a reaction volume of 15 μl containing the following reagents: 7.5 μl of TaqMan PCR Mastermix 2X No UNG (Applied Biosystems, Foster City, CA), 0.75 μl of TaqMan Gene expression assay 20X (Applied Biosystems, Foster City, CA). For each samples 300 nM of primers and 100 nM of Roche probes were used. RT-PCR was carried out on the ABI/Prism 7900 HT Sequence Detector System (Applied Biosystems), using a pre-PCR step of 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The following primers were used:

- Hrs: fwd tcaccacagaagtctcctac; rev ccagggagaattgagga; Stam: fwd gaaatctttgggcagtcg; rev ccagttgctgctgatgtc; Vps25: fwd ccctcaccacccccctaca; rev tgcctgaggtatttgagaaagag; RpL32-RA: fwd cgccatgatgctagagg; rev gcagacactgttgctg;

### Results

The Reported ESCRT-0 Double Mutant Allele Contains a l(2)gl Mutation

To compare the phenotype of the Stam or of the Hrs, Stam double mutant to that of Hrs or of ESCRT-I, -II, -III mutants, we generated clones of cells mutant for Stam2L2896 (Mutant cells are GFP-negative; see Material and Methods) in the follicular epithelium (FE) of the Drosophila ovary. As it is the case of FE cells mutant for HrsD8, Stam mutant FE cells display normal epithelial morphology (Fig. 1A-C). Similarly, we observed no detectable phenotype when we generated mosaic eye imaginal discs (Fig. 1E-G) or eye imaginal discs consisting predominantly of mutant cells (Fig. 1M-O) for both Hrs and Stam. In contrast, cells homozygous for a recently reported HrsD8, Stam2L2896 double mutant allele [23] formed large clones of mesenchymal-like cells (Fig. S1A). Additionally, mosaic eye imaginal discs or eye imaginal discs consisting predominantly of cells mutant for both Hrs and Stam showed a similar loss of epithelial architecture phenotype (Fig. S1B,D).

Both Hrs and Stam are on chromosome 2L, which harbors in a sub-telomeric position the tumor suppressor l(2)gl, a gene frequently lost by spontaneous deletion [29]. Thus, we wondered whether the Hrs, Stam double mutant chromosome present in the Bloomingston stock center carried a mutation in l(2)gl. Failure to complement the null allele l(2)gl indicated a possible lesion in l(2)gl on the chromosome carrying both Hrs and Stam mutations. To test if it was indeed the case, we recombined away the distal part of chromosome 2L containing l(2)gl from the Hrs Stam chromosome and retested for complementation. We isolated several independent recombinants that fail to complement Hrs and Stam deficiencies but complement l(2)gl, a further indication of the presence of l(2)gl mutation in the original Hrs Stam chromosome (Hrs Stam l(2)gl triple mutant henceforth; see Material and Methods).

### ESCRT-0 Components are not Required for Tumor Suppression in Drosophila

To test whether the HrsD8, Stam2L2896 mutant chromosome devoid of the l(2)gl mutation still possessed tumor-promoting ability, we analyzed mosaic FE, mosaic eye discs, or eye discs consisting predominantly of cells mutant for the recombined allele. Interestingly, these do not display loss of tissue architecture (Fig. 1D, H, P), as is the case of single Hrs or Stam mutant alleles, suggesting that the l(2)gl lesion in the original double mutant allele was responsible for the loss of tumor suppression phenotypes. These data indicate that simultaneous loss of both ESCRT-0 components do not lead to loss of tissue architecture, a striking difference to ESCRT-I, -II, -III mutations, which are tumorigenic [11,17,18]. Consistent with this surprising difference, we found that eye discs consisting predominantly of cells mutant for Hrs, or Stam or both Hrs and Stam progress to form adult eyes. These are smaller than wild-type and have a rough appearance but contain some mutant photoreceptors (Fig. 1Q-T). The scarcity of mutant adult photoreceptors might be due to cell death, as we occasionally see apoptotic cells in clones of Hrs, or Stam or both Hrs and Stam double mutants (Fig. 1I-L). In sheer contrast to these, a number of ESCRT-I, -II, -III mutations, such as those mapping to Tsg101, Vps28, Vps25, Vps20, when made homozygous in eye discs, display a Mutant Eye No Ceclosion (MEN) phenotype that have been associated loss of tumor suppression in Drosophila [30]. Overall, these data suggest that the activity of Hrs and Stam is not tumor suppressive in two different Drosophila epithelial tissues.
Figure 3. ESCRT-0 mutants do not affect endosomal maturation. (A) Example of eye tissue almost completely homozygous for a WT (left) or mutant chromosome (right). WT cells are marked with GFP and represent 10% of the disc tissue. (B) Phase contrast images of cross-sections used for EM show the indicative regions used for ultrastructural analysis (pink boxes). Note the absence of monolayer architecture in sections of mutant tissues. (C–E) Electron micrograph of sections of eye disc tissue of the indicated genotype. A portion of the apical part of 2–3 epithelial cells above the level of the basal nuclei is shown. While MVEs (highlighted in red) are absent in Vps25 mutant cells, they are present in ESCRT-0 mutant cells. Quantification of MVE density, diameter, section area and ILV content is presented below each panel. (F–H) Incorporation of Lysotracker in mosaic discs. A single subapical confocal cross-section is shown in each panel, showing no difference in acidification in WT (GFP-positive) versus mutant cells. Labels are as follows: PM: peripodial membrane, DT: disc tissue, LU: Apical lumen, AJ: Adherens Junctions, ER: Endoplasmic Reticulum, GA: Golgi apparatus, MI: Mitochondrium, NU: Nucleus, GV: giant vacuoles, IS: interstitial space between unpolarized cells. doi:10.1371/journal.pone.0093987.g003
Figure 4. ESCRT-0 mutations do not alter Notch signaling in FE cells. (A–F) Mosaic egg chambers at stages 5–7 of oogenesis stained to detect the Notch targets Hnt (A–C) and Cut (D–F) and f-Actin. Stam or Hrs Stam mutant cells are marked by the absence of GFP. In both Stam and Hrs Stam mutant FE cells, Hnt is normally expressed and Cut normally downregulated after stage 6, indicating no impairment of Notch signaling activation. (A’–F’) show single channels.

doi:10.1371/journal.pone.0093987.g004
Impaired ESCRT-0 Activity Leads to Accumulation of Ubiquitin, Notch and Dome

To test whether ESCRT-0 mutants are able to sort ubiquitylated cargoes, we immunostained mosaic eye disc and FE cells containing clones of cells mutant for *Hrs*, or *Stam* or both *Hrs* and *Stam* with an antibody specific to mono- and poly-ubiquitin chains. In contrast to WT cells, but similarly to previous reports of *Hrs* and of ESCRT-I, -II, -III mutants [20–22], *Hrs*, *Stam* and *Hrs Stam* mutant cells, as well as *Hrs Stam* l(2)gl triple mutant cells accumulated ubiquitin (Fig. 2A–F; Fig. S1E–F).

The Notch receptor is a cargo prominently subjected to endosomal sorting in *Drosophila* discs and FE cells [11–13]. To assess whether Notch is sorted and degraded in endosomes of ESCRT-0 mutant cells, we immunolocalized Notch in *Hrs*, *Stam* single or double *Hrs Stam* mutant cells and in *Hrs Stam* l(2)gl triple mutant cells. Compared to WT cells, mutant eye disc cells displayed accumulation of Notch, as assessed with an antibody that recognizes the extracellular domain of Notch (NECD). Accumulation is less evident using an antibody to the intracellular portion (NICD), and is not present in l(2)gl mutant discs (Fig. 2G–K; Fig. S1G, I–K). Similarly, we found accumulation of Domeless (Dome), the single-pass non-tyrosine-kinase receptor for JAK/STAT signaling (Fig. 2L; Fig. S1H).

To follow sorting and degradation of transmembrane proteins over time, we performed a Notch endocytic trafficking assay in living imaginal discs [22]. Briefly, we cultured freshly dissected discs in insect media in presence of a Notch antibody that recognizes an extracellular epitope. We then washed and chased internalization of the bound antibody over time. In contrast to WT discs, but like *Hrs* and *Vps25* mutant discs [22], *Stam* mutant, or *Hrs Stam* l(2)gl triple mutant disc cells displayed various degrees of intracellular signal after a 5 hrs chase, indicating that they fail to degrade endosomal Notch (Fig. 2K; Figure S1J–K). Co-staining of Notch with the early endosomal marker Avalanche (Avl) reveals that ungraded Notch and ubiquitin accumulate for the most part in early endosomes (Fig. 2L; Fig. S1I). Overall, these data are consistent with a general defect in endosomal sorting and degradation of ubiquitylated cargoes, including signaling receptors, in ESCRT-0 mutants.

**ESCR-T-0 is not Required for Endosome Maturation**

Given the accumulation of ubiquitin and of Notch and Dome in ESCRT-0 mutant cells, we next assayed whether mutant cells possess mature endosomes. One aspect of endosome maturation involves formation of ILVs during MVE biogenesis. To test whether ILV formation occurs in ESCRT-0 mutants, we analyzed whether ILV formation occurs in ESCRT-0 mutants. In contrast to WT cells, clones of *Hrs* mutant cells possess acidic endo-lysosomal organelles, we cultured mosaic discs in presence of Lysotracker, a vital dye that concentrates in acidic compartments. Compared with WT cells, clones of *Hrs* mutant cells incorporate equal levels of Lysotracker, consistent with previous evidence [31](Fig. 3E). Similarly, *Stam* or *Hrs Stam* mutant cells are indistinguishable from surrounding WT cells, indicating no impairment of the ability to acidify endocytic organelles (Fig. 3F–G). Taken together, these data suggest that loss of *Hrs*, *Stam* or both do not affect endosomal maturation.

**ESCR-T-0 is not Required for Notch Signaling Activation or Downregulation**

Accumulation of Notch in endosomes of ESCRT-I, -II, -III mutants correlates with ectopic and ligand-independent Notch signaling [18]. In contrast, mutations that disrupt earlier steps of endocytic vesicle trafficking such as those affecting Rab5 and avl inhibit activation of Notch [22]. Therefore, it is unclear what to expect in ESCRT-0 mutants. To assay Notch activation in mutant FE cells, we monitored expression of the transcription factors Hindsight (Hnt) and Cut, whose expression is modulated by Notch activation at mid-oogenesis. In fact, upon expression of the ligand Delta in germline cells at stage 6 of oogenesis, Notch signaling is activated in neighboring FE cells. As a result, FE cells downregulate Cut expression, unregulate Hnt expression, arrest mitotic cell cycles and begin to endoreplicate [32,33]. Surprisingly, the pattern of Hnt and Cut expression detected by immunofluorescence in small clones of *Hrs*, or *Stam*, or *Hrs Stam* mutant FE cells at stage 5–7 was unchanged, when compared to WT cells, indicating that Notch activation is not altered in ESCRT-0 mutants (Fig. 4A–F). In clear contrast, precocious expression of Hnt before stage 6 is observed in ESCRT-I mutant FE cells [22]. In *Hrs Stam* l(2)gl triple mutant cells, ectopic Hnt expression and failure to downregulate Cut expression is visible only in multilayering cells that do not contact the germline and are likely to not be reached by the ligand (Fig. S11–M). Overall, our data confirm and extend the notion that ESCRT-0 activity is not required for Notch signaling and that its loss do not promote ectopic, ligand-independent activation, as observed in other ESCRT-I, -II, -III mutants [18,20,21].

**Discussion**

The ESCRT-0 Complex is Dispensable for Tumor Suppression in *Drosophila*

In this study, we study the effects of impairment of ESCRT-0 function on *Drosophila* epithelial tissue development in vivo. We found that the recently reported [23,24] *Hrs*<sup>l(2)gl</sup> *Stam*<sup>l(2)gl</sup> double mutant allele carries a third mutation in l(2)gl, which we show is responsible for the loss of tumor suppressor (TS) phenotype of triple mutant tissue (Fig. S1). We analyzed independent *Hrs*<sup>l(2)gl</sup> *Stam*<sup>l(2)gl</sup> recombinants devoid of l(2)gl mutations and observed that, when in homozygosity, these do not possess ability to growth into neoplasms, indicating that ESCRT-0 function *per se* is not tumor suppressive in *Drosophila*. While it is not clear when the reported *Hrs*<sup>l(2)gl</sup> *Stam*<sup>l(2)gl</sup> chromosome acquired the previously unrecognized l(2)gl mutation and whether some of the phenotypes
reported in the literature are due to impairment of \( l(2)gl \) activity [23], we advise future use of our recombinant chromosome devoid of the \( l(2)gl \) lesion.

The fact that ESCRT-0 is dispensable for tumor suppression (TS) marks a striking difference to mutations in most genes encoding components of the downstream ESCRT-I, -II, -III complexes. The discrepancy between the tissue architecture phenotypes of ESCRT-0 and other ESCRTs could be explained by different scenarios that we discuss below.

1) ESCRT TS function is not linked to endosomal sorting: ESCRT-III is very ancient, it is present in archaea and unicellular organisms [34], in which its membrane bending capacity is mostly used in the last step of cytokinesis [35,36]. In contrast, ESCRT-0 has evolved recently, is dispensable for completion of cytokinesis, and might represent a specialization to sort a subset of cargoes in endosomes [37]. However, we do not favor the idea that the tumor suppression activity of ESCRT complexes correlates with their involvement in cytokinesis. In fact, while cell division and cytokinesis defects have been extensively linked to tumorigenesis, ESCRT-II, another ESCRT complex that behaves as TS, is dispensable for cytokinesis [35,36].

2) ESCRT TS function is linked to endosomal sorting and residual Hrs or Stam function might be present in mutants: We think this is unlikely because both \( Hrs^{229} \) and \( Stam^{229} \) are null alleles to the best of our knowledge. In fact, \( Hrs \) expresses only the amino terminal first quarter of the protein, and is devoid of most functional domains [20], while \( Stam^{229} \) line harbors a non sense mutation leading to an early stop codon at amino acid 6 [24]. Both genes have no paralogs in \( Drosophila \). In addition, quantitative RT-PCR also shows that in both \( Hrs \) and \( Stam \) \( l(2)gl \) mutants only 50% of the \( Hrs \) transcript is present. In both \( Stam \) and \( Hrs \) \( l(2)gl \) mutant tissues, only 20–30% of the \( Stam \) transcript is present (Fig. S2), indicating that in either backgrounds both mutant \( Hrs \) and \( Stam \) transcripts are possibly subjected to non sense-mediated decay, and further decreasing the likelihood of residual function.

3) ESCRT TS function is linked to endosomal sorting, but the relevant cargoes do not require Hrs or Stam for their sorting: Several studies suggested that alternative ESCRT-0 proteins may work in parallel, or even instead of Hrs and Stam. Good candidates are two families of proteins, GGA and Tom1 (target of Myb1), both present in \( Drosophila \), that have similar characteristics to those of ESCRT-0 components. These in fact contain VHS, Ubiquitin binding, and Clathrin binding domains typical of ESCRT-0 components, they recruit ubiquitylated proteins to endosomal membranes, and they interact with ESCRT-I and Clathrin [38–40]. Thus, ESCRT-0 complex could be dispensable for sorting of TS-relevant proteins. Interestingly, endocytosis of junctional adhesion proteins, such as E-Cadherin, directly regulates polarity in \( Drosophila \) epithelia [41]. Consistent with a minor role of ESCRT-0 in controlling polarity, a study showed that mutation in \( Drosophila \) \( Hs \) does not affect the localization of DE-Cadherin [21]. In contrast, junctional adhesion proteins appear sensitive to function of more downstream ESCRTs. Indeed, ESCRT-I and -III have been shown to be required for degradation of adhesive molecules, such as Claudin-1, and for maintenance of polarity in vertebrate epithelial cells [42]. Thus, we predict that proteins that play a role in ensuring correct epithelial architecture and polarity, such as those involved in cell-cell adhesion, might not require ESCRT-0 for their sorting and degradation.

**ESCRT-0 is Dispensable for Ectopic Notch Activation in Endosomes**

In \( Stam \) and double mutants we observed accumulation of Notch receptor in endosomes, especially when immunolocalizing with an anti-Notch ECD, which recognizes the extracellular portion of Notch. It is not clear why the accumulation is less evident by immunolocalization of the intracellular portion of Notch with anti-Notch ICD. It is possible that either the latter accumulated less than the former, perhaps due to the fact that Notch is normally activated in mutant cells, or the two antibodies possess different efficiency in recognizing their epitopes. However the case, we were surprised to find no ectopic activation of Notch signaling. A trivial possibility to explain the difference with ESCRT-I, -II, -III mutants is that Notch trafficking and degradation might be quantitatively less affected than in ESCRT-0 mutant. Although our assays are not quantitative, the genetic nature of the ESCRT-0 mutants renders this possibility rather unlikely.

Another possibility is that recycling from endosomes to the plasma membrane might be important to prevent ectopic Notch receptor activation and recycling might still functioning in ESCRT-0 mutants, however it might not in ESCRT-I, -II, -III mutants. At present, whether the Notch receptor is recycled to the plasma membrane, and the status of recycling on different ESCRT mutant in \( Drosophila \) epithelia are unknown, preventing us to conclude on the likelihood of such an hypothesis. However, we observe an accumulation of NEDD in endosomes of ESCRT-0 mutants that is comparable to that of ESCRT-I, -II, -III, an evidence that appears to contrast with the possibility of substantial recycling of Notch in ESCRT-0 mutants.

Finally, Notch accumulation in endosomes in ESCRT-0 mutants might not yield ectopic activation because such forced and ligand-independent Notch activation might require the cargo clustering by ESCRT-0 on the limiting membrane of endosomes. Alternatively, endosomes of ESCRT-0 mutant cells might not be mature enough to permit ligand-independent activation. These two not necessarily mutually exclusive possibilities are supported by the fact that Hrs and Stam act with Clathrin to trap and concentrate cargos to be degraded on the endosomal membrane [9], and by evidence in \( Drosophila \) suggesting that ligand-independent Notch activation occurs in late endosome/lysosomes and depends on endosome acidification and maturation [31,43–46]. Our data clearly indicate that two aspects of endosomal maturation, MVE biogenesis and endolysosomal acidification occur normally in ESCRT-0 mutants, suggesting that these could support later events required for ectopic Notch activation. The fact that ectopic Notch activation is not observed in the mutants thus points to cargo clustering as a potential prerequisite for ectopic activation of Notch. Whether cargo clustering is required for efficient Notch cleavage requires further studies.

In summary, our comparative analysis of \( Hrs \) and \( Stam \) in epithelial tissue \textit{in vivo} reveals unexpectedly that ESCRT-0 is dispensable for control of cell polarity and proliferation, a major tumor suppressive event. Our data, marking a striking difference with ESCRT-I, -II, -III components, which act as TS, predict that specific cargoes important for cell polarity are sorted in endosomes independent of ESCRT-0 function, and that Notch activation, on the contrary, might be highly sensitive to receptor clustering by ESCRT-0 on the endosomal membrane.
Supporting Information

Figure S1 l(2)gl is responsible for the loss of tumor suppression phenotype in triple mutants. (A–B) Epithelial disorganization in Hrs, Stam l(2)gl triple mutant tissues revealed by staining to detect sub-cortical f-Aktin. FE cells homogous for Hrs, Stam l(2)gl (marked by the absence of GFP) in a stage 5–6 egg chamber are misshapen and multilayered. Eye imaginal cells homogous for the mutations also show mesenchymal-like cells and autonomous disruption of epithelial organization. (C) Mosaic eye imaginal discs stained with antibody anti-activated Caspase 3 to mark apoptotic cells. In Hrs Stam l(2)gl mutant tissue (GFP negative) apoptosis is activated. (D) Eye imaginal disc formed by mutant cells homogous for Hrs, Stam l(2)gl stained to detect sub-cortical f-Aktin show a tumor-like phenotype. (E–H) Hrs Stam l(2)gl mosaic eye and FE cells (marked by the absence of GFP) stained to detect ubiquitin, Notch and the Domeless receptors. Separate channels are shown in E’F’G’H’. Cells homogous for Hrs Stam l(2)gl show accumulation of ubiquitin, Notch and Domeless receptors. Separate cortical f-Actin show a tumor-like phenotype. (E–H) mutant cells homozygous for (0 hrs). (N–O) Mosaic egg chambers at stages 5–7 of oogenesis stained for Hnt and Cut. Hrs Stam l(2)gl homozygous cells are marked by the absence of GFP. In Hrs, Stam l(2)gl triple mutant cells Hnt expression and failure to downregulate Cut expression is visible only in multilayering cells that are likely not reached by the ligand, indicating that Notch activation is not affected in mutant cells that are exposed to the ligand and Notch is not ectopically activated in those that are not. WT controls for all panels are presented in Fig. 1–2, 4.

Figure S2 Mutant transcripts for Hrs and Stam are subjected to non sense-mediated decay. Quantitative RT-PCR experiment on mRNA extracted from eye imaginal discs from single Hrs or Stam or double Hrs, Stam or triple Hrs, Stam, l(2)gl mutant tissue compared to control indicates reduction of Hrs or Stam mRNA expression in corresponding mutant extracts.

Acknowledgments

We thank Stephane Noselli, the BDSC and the DHSB for reagents, and Simona Polo for critically reading the manuscript.

Author Contributions

Conceived and designed the experiments: TV. Performed the experiments: ET NW KC. Analyzed the data: TV ET NW KC CT. Wrote the paper: Simona Polo for critically reading the manuscript.

References

1. Sigismund S, Confalonieri S, Ciliberto A, Polo S, Scita G, et al. (2012) Endocytosis and signaling: cell logistics shape the eukaryotic cell plan. Physiological reviews 92: 273–366.
2. Milliman I, Vardeny Y (2013) Endocytosis and cancer. Cold Spring Harbor perspectives in biology 5: a016949.
3. Rabst M, Katzmann D, Estate-Sabal E, Mierloof T, Emr SD (2002) Escrt-III: an endosome-associated heterooligomeric protein complex required for meb sorting. Developmental cell 3: 271–282.
4. Rabst M, Katzmann D, Snyder W, Wendland B, Emr SD (2002) Endosome-associated complex, ESCRT-II, recruits transport machinery for protein sorting at the multivesicular body. Developmental cell 3: 283–292.
5. Katzmann D, Babst M, Emr SD (2001) Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell 106: 145–155.
6. Babst M, Stefan C, Rabst M, Emr SD (2003) Vps27 recruits ESCRT machinery to endosomes during MVB sorting. The Journal of cell biology 162: 413–423.
7. Bilodeau PS, Urbanowski JL, Winistorfer SC, Piper RC (2002) The Vps27p–Hse1p complex binds ubiquitin and mediates endosomal protein sorting. Nature Cell Biology 4: 887–898.
8. Piper R, Cooper A, Yang H, Stevens T (1995) VPS27 controls vacuolar and endocytic traffic through a prevacuolar compartment in Saccharomyces cerevisiae. The Journal of cell biology 131: 603–617.
9. Sacher M, Urbé S, Oorschot V, Strous GJ, Klumperman J (2002) Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes. Molecular biology of the cell 13: 1313–1329.
10. Rusten TE, Vaccari T, Stemmark H (2012) Shaping development with ESCRTs. Nature Cell Biology 14: 38–45.
11. Vaccari T, Bider D (2008) Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. The journal of cell biology 119: 755–762.
12. Chuang-Deladale H, Jung AC, Barr MM, Lin L, Payne F, et al. (2010) The Hrs/Stam complex acts as a positive and negative regulator of RTK signaling during Drosophila development. PLoS ONE 5: e10245.
13. Chuang-Deladale H, Jung AC, Lin L, Barr MM, Böhme A, et al. (2007) A genetic mosaic analysis with a repressible cell marker to identify genes involved in tracheal cell migration during Drosophila air sac morphogenesis. Genetics 176: 2171–2187.
14. Newsome T, Ailing B, Dickson BJ (2000) Analysis of Drosophila photoreceptor axon guidance in eyspecific mosaics. Development 127: 831–860.
15. Tapon N, Ito N, Dickson BJ, Treisman J, Hariharan I (2001) The Drosophila tuberous sclerosis complex gene homolog restrict cell growth and cell proliferation. Cell 105: 345–355.
16. Lee T, Luo L (2001) Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. Trends Neurosci 24: 251–254.
17. Goemtoro LA, Yakoby N, Goodhouse J, Schupbach T, Shvartsman SY (2006) Quantitative analysis of the GABA/PUAS system in Drosophila oogenesis. Genetics 44: 66–74.
18. Roegiers F, Kavalier J, Tolwinski N, Chou Y-T, Duan H, et al. (2009) Frequent Unanticipated Alleles of lethal giant larvae in Drosophila Second Chromosome Stocks. Genetics 182: 407–410.
19. Menut L, Vaccari T, Dionne H, Hill J, Wu G, et al. (2007) A mosaic genetic screen for Drosophila neoplastic tumor suppressor genes based on defective puation. Genetics 177: 1667–1677.
31. Vaccari T, Duchi S, Cortese K, Tacchetti C, Bilder D (2010) The vacuolar ATPase is required for physiological as well as pathological activation of the Notch receptor. Development 137: 1825–1832.
32. Deng W, Althauser C, Ruohola-Baker H (2001) Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in Drosophila follicle cells. Development (Cambridge, England) 128: 4737–4746.
33. Lopez-Schier H, St Johnston D (2001) Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. Genes & development 15: 1393–1405.
34. Samson RY, Ohita T, Freund SM, Williams RL, Bell SD (2008) A role for the ESCRT system in cell division in archaea. Science (New York, NY) 322: 1710–1713.
35. Carlton JG, Martin-Serrano J (2007) Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science (New York, NY) 316: 1908–1912.
36. Morita E, Sandrin V, Chung H-Y, Morham SG, Gygi SP, et al. (2007) Human ESCRT and ALIX proteins interact with proteins of the midbody and function in cytokinesis. The EMBO journal 26: 4215–4227.
37. Leung K, Dacks J, Field M (2008) Evolution of the multivesicular body ESCRT machinery; retention across the eukaryotic lineage. Traffic (Copenhagen, Denmark).
38. Blanc C, Charette SJ, Mattei S, Aubey L, Smith EW, et al. (2009) Dictyostelium Tom1 participates to an ancestral ESCRT-0 complex. Traffic (Copenhagen, Denmark) 10: 161–171.
39. Katoh Y, Shibay, Mituhashi H, Yanagida Y, Takatsu H, et al. (2004) Tollip and Tom1 form a complex and recruit ubiquitin-conjugated proteins onto early endosomes. The Journal of biological chemistry 279: 24435–24443.
40. Gullapalli A, Wolfe BL, Griffin CT, Magunson T, Trejos JA (2006) An essential role for SNX1 in lysosomal sorting of protease-activated receptor-1: Evidence for retromer, Hrs, and Tsg101-dependent functions of sorting nexins. Molecular biology of the cell 17: 1228–1238.
41. Leibfried A, Fricke R, Morgan MJ, Bogdan S, Balleix Y (2008) Drosophila Cip4 and WAP1 define a branch of the Cdc42-Par6-aPKC pathway regulating E-cadherin endocytosis. Current Biology 18: 1639–1648.
42. Yousafian J, Troost T, Grosee F, Sasamura T, Fortini M, et al. (2013) Dmon1 controls recruitment of Rab7 to maturing endosomes in Drosophila. Journal of cell science 126: 1583–1594.