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Sarah E. Woodfield
The University of Texas MD Anderson Cancer Center

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De-Regulation of JNK and JAK/STAT Signaling in ESCRT-II Mutant Tissues Cooperatively Contributes to Neoplastic Tumorigenesis

Sarah E. Woodfield1,2, Hillary K. Graves2, Jacob A. Hernandez2, Andreas Bergmann1,2,3*

1 Graduate Program in Developmental Biology, Baylor College of Medicine, Houston, Texas, United States of America, 2 Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas, United States of America, 3 Department of Cancer Biology, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America

Abstract

Multiple genes involved in endocytosis and endosomal protein trafficking in Drosophila have been shown to function as neoplastic tumor suppressor genes (nTSGs), including Endosomal Sorting Complex Required for Transport-II (ESCRT-II) components vacuolar protein sorting 22 (vps22), vps25, and vps36. However, most studies of endocytic nTSGs have been done in mosaic tissues containing both mutant and non-mutant populations of cells, and interactions among mutant and non-mutant cells greatly influence the final phenotype. Thus, the true autonomous phenotype of tissues mutant for endocytic nTSGs remains unclear. Here, we show that tissues predominantly mutant for ESCRT-II components display characteristics of neoplastic transformation and then undergo apoptosis. These neoplastic tissues show upregulation of c-Jun N-terminal Kinase (JNK), Notch, and Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling. Significantly, while inhibition of JNK signaling in mutant tissues partially inhibits proliferation, inhibition of JAK/STAT signaling rescues other aspects of the neoplastic phenotype. This is the first rigorous study of tissues predominantly mutant for endocytic nTSGs and provides clear evidence for cooperation among de-regulated signaling pathways leading to tumorigenesis.

Introduction

Tumor development involves destabilization of the well-controlled processes of cell proliferation, cell polarization, and programmed cell death that are tightly regulated by widely conserved signaling pathways. Therefore, genes that act as regulators of these signaling pathways may behave as nTSGs. In Drosophila, as well as in other organisms, genes that control endocytosis and endosomal protein sorting behave as nTSGs. Such endocytic nTSGs include avalanche (avl) [1], Rab5 [1], tspl[5] [2], Rabenosyn (Rbsn) [2], tumor suppressor proteins 101 (tsg101 aka erupted (ept)) or vps22) [3], vps28 [4], vps25 [5–7], vps22 (aka larsen (lars)) [8], vps20 [4], shrub (shub) [4], vps2 [4], and vps4 [9]. These endocytic nTSGs are involved in endocytosis and endosomal protein sorting of cell signaling receptors and other membrane proteins and inhibit tumor formation by ensuring proper trafficking and collection of cargoes that function in growth control, cell survival, and apical-basal polarity in epithelial tissues.

The ESCRT machinery promotes the maturation of early endosomes into multi-vesicular bodies (MVBs) [10–12]. This is a complex process that involves four ESCRT complexes, ESCRT-0, -I, -II and -III. Of interest to this study are the ESCRT-II components vps22, vps25, and vps36. The products of these genes mediate the transfer of cargo from ESCRT-I to ESCRT-III [10–12]. Loss-of-function mutations of these genes block this process, which causes abnormal signaling and triggers a complex phenotype composed of autonomous and non-cell autonomous effects [5–8].

Previous studies of the mutant phenotypes of ESCRT-II components and other endocytic nTSGs focused on their mosaic phenotype, when mutant clones are surrounded by wild-type cells. Thus, the complex mosaic phenotype of endocytic nTSGs has been well characterized. Epithelial polarity and proliferation control are disrupted in mutant clones [1,3,5,7,9]. Mutant clones in eye-antennal imaginal discs fail to express the neuronal marker ELAV, indicating that they fail to differentiate [6,7]. A clear non-cell autonomous effect of mutant clones on proliferation is observed in tissues mosaic for tsg101, vps22, or vps25 [3,5,7,8]. The non-mutant tissues surrounding the mutant clones display increased proliferation [3,5,7]. Such tissues form multilayered discs and overgrown adult structures [1,3,5,7]. vps25 mutant clones also promote non-cell autonomous cell survival through upregulation of the apoptosis inhibitor Diap1 [3,5,8,13].

In mutant clones of endocytic nTSGs, endosomal trafficking is blocked and membrane proteins accumulate in abnormal endosomal compartments [1,3,5,9,14]. For example, Notch protein accumulates in abnormally enlarged early endosomes where it undergoes ligand-independent processing and activation [1–8]. Active Notch induces non-cell autonomous proliferation in vps22,
**Materials and Methods**

**Drosophila Genetics and Generation of Predominantly Mutant Imaginal Discs**

The following mutants and transgenic lines were used: *vps22*^{F88.4} [20], *vps25*^{F88.6} [3], *vps36*^{F88.6} (this study), *nkd^{H166} [21], Stat92E^{F79} [22], *pw-lacZ [23], Gbe-Su(H)-lacZ [24], Bicoid^{b} 2.6-lacZ [25], *10X-STAT-GFP [26], UAS-betatubulin^{GFP} [27], and *sy-Gal4^{F20} [29], *vps36*^{F88.6} is a null allele generated by imprecise excision of the P-element transposon inserted in the first exon 29 base pairs upstream of the initiator ATG in the *vps36*^{F88.6} allele.

To generate imaginal disc predominantly mutant for *vps22*, *vps25*, or *vps36*, we used the *sy-FLP/c1* technique [19,28]. *cl* indicates an anonymous cell lethal mutation that kills cells when homozygous [19,28]. The **ESCRT-II** mutant alleles were crossed to *sy-FLP/FRT cl flies*. The use of the *FRT* depended on the location of the **ESCRT-II** gene in the genome. The complete genotypes are indicated in the legends to the figures.

**Immunohistochemistry**

Imaginal discs were dissected from third instar larvae and stained using standard protocols. The following antibodies were used: mouse α-Dlg (1:20); rat α-ELAV (1:40), mouse α-Mmp1 (catalytic domain; 1:50), and mouse α-Notch^{Netra} (1:20; DSHB, University of Iowa); mouse α-BrdU (1:50; Becton Dickinson); rabbit α-cleaved Caspase-3 (Casp-3*; 1:500; Cell Signaling Technology); mouse α-β-gal (1:1,000) and rabbit α-galNK (1:100; Promega); and rabbit α-PKCl (1:100; Santa Cruz Biotechnology). AF488-phalloidin and AF546-phalloidin were obtained from Sigma Aldrich. Cy-3 and Cy-5 fluorescein-conjugated secondary antibodies were obtained from Jackson Immunoresearch. Vectashield with DAPI was obtained from Vector Laboratories. TUNEL kit was obtained from Roche Diagnostics. Images were captured using Olympus Optical FV500 or FV1000 confocal microscopes and processed using Adobe Photoshop CS4.

**Results**

**ESCRT-II Mutant Tissues Show Neoplastic Characteristics**

The *sy-FLP/c1* method generates eye-antennal imaginal discs that are almost entirely composed of mutant tissue in otherwise heterozygous animals (see Methods) [19,28]. This is accomplished by elimination of the twin-spots after *sy-FLP*-induced mitotic recombination by a cell lethal (*cl*) mutation that is present on the homologous chromosome arm. The use of the *sy-FLP* ensures high FLP activity such that most cells undergo mitotic recombination and only a few heterozygous cells remain. Thus, eye-antennal discs generated by this method are almost entirely mutant for the gene of interest.

We used the *sy-FLP/c1* system to generate tissues predominantly mutant for **ESCRT-II** components *vps22*, *vps25*, or *vps36*. These predominantly mutant epithelial tissues have a very striking phenotype: unlike wild-type single-layered eye-antennal imaginal discs, they overgrow into multi-layered, dense “balls” of cells (Figure 1) [7,8,17]. These discs also vary considerably in size. Some are about the size of wild-type discs or even slightly smaller while others can be three to five times as large [8,17]. This was also reported for other endocytic nTSGs [21,27]. To understand this tumor-like phenotype in more detail, we performed proliferation, cellular architecture, differentiation, and metastatic potential of eye-antennal discs predominantly mutant for *vps22*, *vps25*, or *vps36*.

To assay proliferation in the predominantly mutant tissues, we used Bromodeoxyuridine (BrDU) labeling to mark cells in S-phase.
Figure 1. Tissues predominantly mutant for ESCRT-II components vps22, vps25, or vps36 show neoplastic characteristics. Shown are predominantly mutant eye-antennal imaginal discs. Phalloidin (green) is used to mark the overall shape of the tissue. Scale bars represent 50 μm. (A–D) BrdU (red and grayscale) labelings show that proliferation is increased in discs predominantly mutant for vps22 (B,B'), vps25 (C,C'), or vps36 (D,D') as compared to proliferation in control discs (A,A'). aPKC (red and grayscale (E',F',G',H')) and Dlg (green and grayscale (E'',F'',G'',H'')) labelings of discs predominantly mutant for vps22 (F–F''), vps25 (G–G''), or vps36 (H–H'') show that cellular architecture is disrupted, as compared to the architecture of control discs (E–E''). (I–L) ELAV (red and grayscale) labelings of discs predominantly mutant for vps22 (I,J), vps25 (K,K'), or vps36 (L,L') show that very few cells in the mutant discs differentiate normally, as compared to differentiation in control discs (I,I'). (M–P) Mmp1 (red and grayscale) labelings of discs predominantly mutant for vps22 (M,N), vps25 (O,O'), or vps36 (P,P') show that levels of this protein are elevated, as compared to Mmp1 levels in control discs (M,M'). Genotypes: (A) eyFLP; FRT1828/FRT1828 cl k FRT42D y eyFLP; FRT42D y /FRT42D cl. (B,F,J,N) eyFLP; FRT828 vps223560 y/FRT828 cl. (C,G,K,O) eyFLP; FRT42D vps253560 y /FRT42D cl. (D,H,L,P) eyFLP; vps36480 FRT80B/c FRT80B.

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Control discs show the normal BrdU pattern in eye-antennal discs (Figure 1A). Of note is the posterior part of the eye disc in which cells are post-mitotic and differentiate into photoreceptor neurons, cone cells, and other cell types. In discs predominantly mutant for ESCRT-II components, BrdU labeling indicates that proliferation is occurring at elevated levels throughout the entire disc (Figure 1B–D). Post-mitotic areas are not visible or are very small. Thus, proliferation is up-regulated in tissues predominantly mutant for vps22, vps25, or vps36.

To examine cellular architecture of tissues predominantly mutant for ESCRT-II components, we first labeled discs with phalloidin. Phalloidin recognizes cortical actin and thus highlights cellular architecture and organization throughout tissues. Control discs stained with phalloidin show a consistent shape characteristic of Drosophila eye-antennal imaginal discs (Figure 1A,J,M). discs predominantly mutant for ESCRT-II components trade this characteristic shape for a disorganized, expanded, amorphic structure in which the antennal and eye portions of the disc cannot be distinguished (Figure 1B,J–L,N–P). Next, to specifically examine epithelial polarity, we labeled predominantly mutant tissues with antibodies recognizing atypical Protein Kinase C (aPKC) and Discs large (Dlg). In control tissue, aPKC localizes to the polarized apical membrane domain while Dlg is found at the basolateral membrane domain (Figure 1E). In the predominantly mutant tissues, aPKC and Dlg are spread outside of their respective regions of wild-type localization (Figure 1F–H), indicating that apical-basal polarity is disrupted. Together, these data indicate that cellular architecture is disrupted in vps22, vps25, and vps36 mutant tissues, which is consistent with previous reports [7,8,17].

It has been shown previously that clones of vps25 mutant cells in mosaics fail to differentiate [6,7]. Therefore, we were curious to examine the ability of cells to differentiate if almost the entire eye-antennal disc is mutant. Photoreceptor neurons are the first cells that differentiate during eye development. Using ELAV as a neuronal marker, we labeled eye-antennal discs almost entirely mutant for vps22 (N,N), vps25 (O,O'), or vps36 (P,P') show that levels of this protein are elevated, as compared to Mmp1 levels in control discs (M,M'). Genotypes: (A) eyFLP; FRT1828/FRT1828 cl k FRT42D y eyFLP; FRT42D y /FRT42D cl. (B,F,J,N) eyFLP; FRT828 vps223560 y/FRT828 cl. (C,G,K,O) eyFLP; FRT42D vps253560 y /FRT42D cl. (D,H,L,P) eyFLP; vps36480 FRT80B/c FRT80B.

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Loss of epithelial integrity and apical-basal polarity, increased proliferation, and loss of differentiation are hallmarks of neoplastic transformation. It has also been demonstrated that vps25 mutant cells have invasive behavior [6]. Matrix metalloprotease 1 (Mmp1) remodels the extracellular matrix and is known to be elevated in and required for metastasis of Drosophila tumors [29]. Therefore, to correlate the metastatic potential of the predominantly mutant vps22, vps25, and vps36 discs with Mmp1 expression, we labeled these discs with an antibody recognizing Mmp1. In control eye-
antennal imaginal discs, Mmp1 is present at very low levels (Figure 1M). In contrast, in the predominantly mutant discs, Mmp1 is present at high levels throughout the discs (Figure 1N–P).

Taken together, these data demonstrate that ESCRT-II components vps22, vps25, and vps36 are strong nTSGs and that eye-antennal imaginal discs predominantly mutant for these genes display neoplastic characteristics.

**Notch, JAK/STAT, and JNK Signaling are Up-regulated in ESCRT-II Mutant Tissues**

Due to the endosomal sorting defect in ESCRT-II mutant tissues, multiple signaling pathways are de-regulated [4–8,14,15]. In discs mosaic for ESCRT-II mutants, it is well understood how de-regulation of signaling contributes to the non-cell autonomous proliferation and survival phenotypes (see Introduction). However, these studies in mosaic tissues fail to answer two important questions: (1) What signaling pathways are de-regulated in predominantly mutant tissues completely independent from interactions with non-mutant populations of cells? (2) Does this autonomous de-regulation of signaling contribute to the autonomous neoplastic phenotype? To answer the first question, we examined levels of Notch, JAK/STAT, and JNK signaling in discs predominantly mutant for ESCRT-II components.

Multiple studies have shown that Notch signaling is up-regulated in tissues mosaic for ESCRT components [3–5,7,8,14]. Thus, we were interested to examine levels of the Notch signaling pathway in tissues predominantly mutant for ESCRT-II components. To assess Notch signaling, we used two Notch reporters, the Gbe-Su(H)-lacZ reporter [24] and the E(spl)m8 2.61-lacZ reporter [25]. In control discs, Notch signaling is high in a very stereotypical pattern in the posterior of the eye disc and in the antennal disc (Figure 2A,B). Use of the Gbe-Su(H)-lacZ reporter in vps25 mutant discs showed that Notch signaling is very high throughout the entire disc (Figure 2D). We used the E(spl)-lacZ reporter to examine Notch activity in vps22 and vps36 mutant tissues and found that Notch signaling is indeed very high but only in about half of each mutant disc (Figure 2C,E). To further examine Notch signaling within mutant discs, we assayed levels of the Notch protein using an antibody that recognizes the intracellular portion of the receptor. We found that protein levels are indeed very high throughout mutant discs (Figure S1B–D), supporting the results found with the Gbe-Su(H)-lacZ reporter. From these data, we clearly see that Notch signaling is up-regulated in tissues predominantly mutant for ESCRT-II components.

In genetic mosaics, increased JAK/STAT signaling has been observed in tsg101 and vps25 mutant clones, and Notch-induced upregulation of the JAK/STAT ligand Upd has been shown to contribute to the non-cell autonomous increase of proliferation in neighboring non-mutant cells [3,5,7,8,18]. Thus, we were interested to see if JAK/STAT signaling is affected autonomously in predominantly ESCRT-II mutant tissues. To assess levels of JAK/STAT signaling, we used the well-characterized 10X-STAT-GFP reporter [26]. In control discs, JAK/STAT signaling is only active in the posterior portion of the eye disc and in the antennal disc (Figure 2F). In contrast, JAK/STAT signaling is clearly very elevated throughout ESCRT-II mutant discs (Figure 2G–I).

One additional pathway that is autonomously induced in mutant clones of endocytic nTSG mosaics is JNK signaling [5,9,15,16,30]. It is assumed that JNK signaling is induced by cell competition between mutant (loser) and non-mutant (winner) cells in the mosaics. In discs predominantly mutant for ESCRT-II genes, the competitive interaction between mutant and non-mutant tissue is removed because most of the non-mutant tissue is eliminated and only mutant tissue remains. We were therefore surprised to see strong labeling with the pJNK antibody, which detects phosphorylated and thus activated JNK, in discs predominantly mutant for ESCRT-II components compared to controls (Figure 2J–M). We also observed a strong induction of Gbe-lacZ, a JNK-reporter transgene, in discs predominantly mutant for vps25 (Figure S2B). Therefore, JNK activity is induced in ESCRT-II mutant discs independently of cell competition. Taken together, these data show that the Notch, JAK/STAT, and JNK signaling pathways are up-regulated in predominantly ESCRT-II mutant tissues and support a possible role for these conserved signaling pathways in the neoplastic phenotype observed in these tissues.

**Tissues Predominantly Mutant for ESCRT-II Components are Apoptotic**

JNK signaling in nTSG mutant clones in mosaic discs triggers apoptosis [5,16,30,31]. Thus, although competitive interactions are largely abolished in predominantly ESCRT-II mutant discs, which are often overgrown, we examined these discs for apoptosis. We assayed cell death by cleaved Caspase-3 (Cas-3*) and TUNEL labeling in predominantly mutant discs. In control discs, a few Cas-3* positive cells are scattered throughout the tissue, but most cells are not apoptotic (Figure 3A). However, surprisingly, discs predominantly mutant for ESCRT-II genes show high levels of Cas-3* throughout (Figure 3B–D). Similar results were obtained with TUNEL labeling (Figure 3E–H), which detects DNA fragmentation, a hallmark of apoptosis [32], indicating that apoptosis is indeed occurring. Taken together, although competitive interactions between mutant and non-mutant cells are eliminated in discs predominantly mutant for ESCRT-II components, they display high levels of apoptosis.

So far, we have analyzed the phenotypes of eye-antennal imaginal discs of ESCRT-II mutants of third instar larvae. We also observed that animals with eye-antennal imaginal discs predominantly mutant for ESCRT-II components die as pharate pupae. Based on our data from imaginal discs, we hypothesized that the apoptosis of the discs may contribute to the death of the pharate pupae. Dissection and examination of the pharate pupae demonstrated that they lack head structures (Figure 4A,B). Thus, it is likely that the apoptosis of the mutant tissues is leading to the death of the animal.

**Inhibition of JNK Affects the Neoplastic Transformation of ESCRT-II Mutant Tissues**

We were curious to examine the role of apoptosis and JNK signaling in these discs. JNK is particularly interesting in this respect because under certain conditions it not only induces apoptosis, but also non-cell autonomous proliferation [33–35]. Therefore, we blocked apoptosis and JNK signaling in these mutant tissues and examined the contribution of these pathways to the neoplastic phenotype of imaginal discs predominantly mutant for ESCRT-II components.

We first blocked apoptosis in mutant discs by generating discs that are predominantly double mutant for vps25 and ark, the Apaf-1 related killer in flies that is an essential component of the cell death pathway [21,36–40]. In vps25 ark double mutant discs, cell death is completely inhibited, as shown by Cas-3* labeling (Figure 5B). In these double mutant discs, the neoplastic phenotype is even more severe. In some animals, the two eye-antennal imaginal discs fuse together into one large epithelial mass (Figure 5F); in a few instances, the two brain lobes and two discs fuse together into a large mass. These tissue fusions were not observed in vps25 single mutant discs and may indicate even more
invasive behavior of apoptosis-inhibited vps25 mutant tissue. High levels of proliferation, as indicated by BrdU incorporation, are consistent throughout the entire predominantly mutant tissues (Figure 5D). Cellular architecture is completely disrupted, as shown by the drastic spreading of aPKC and Dlg localization (Figure 5F). A few cells differentiate normally and thus are positive for ELAV, but most cells fail to differentiate (Figure 5H). Finally, there are high levels of Mmp1 throughout the mutant tissue, indicating that the tissue has the potential to be invasive (Figure 5J). Importantly, eye-antennal imaginal discs predominantly mutant for ark alone do not show any neoplastic characteristics (Figure 5C,E,G). Therefore, it is clear that cell death is not required for neoplastic transformation in tissues predominantly mutant for ESCRT-II components. In contrast, since the phenotype of vps25 ark double mutant discs is more severe than that of vps25 single mutant discs (Figure 1), apoptosis in these mutant discs serves as a tumor suppressor mechanism to eliminate the cancerous tissue.

We also examined the role of JNK signaling in apoptosis, proliferation and neoplastic characteristics in discs predominantly mutant for ESCRT-II genes by inhibiting JNK signaling through overexpression of a dominant negative form of the Drosophila JNK homologue basket (bsk), bskDN [27], using ey-Gal4. In control discs, overexpression of bskDN in otherwise wild-type discs has no apparent effect on architecture, polarity, differentiation, and Mmp1 expression (Figure 5K,M, Figure S3A,C,E). However, compared to the apoptosis observed in vps25 mutant discs (Figure 3C,G), TUNEL-positive cell death is strongly suppressed by expression of bskDN in discs predominantly mutant for vps25 (Figure 5L) suggesting that JNK signaling contributes to the apoptotic phenotype of predominantly mutant ESCRT-II eye discs. Intriguingly, the proliferation pattern is also reduced in these discs.
as assayed by BrdU labeling (Figure 5N), implying that JNK-induced proliferation at least partially contributes to the strong proliferation phenotype of \(vps25\) mutant discs. Labeling with phalloidin (green) or DAPI (green) is used to mark the overall shape of the tissue. Scale bars represent 50 \(\mu\)m. (A–D) Cleaved Caspase-3 (Cas-3*; red and grayscale) labelings show that apoptosis is increased in discs predominantly mutant for \(vps22\) (B), \(vps25\) (C), or \(vps36\) (D), as compared to apoptosis in control discs (A,A'). (E–H) TUNEL (red and grayscale) labelings show that apoptosis is increased in discs predominantly mutant for \(vps22\) (F), \(vps25\) (G), or \(vps36\) (H), as compared to apoptosis in control discs (E,E'). Genotypes: (A) eyFLP; FRT42D y'\(^{-}\)/FRT42D cl. (B,F) eyFLP; FRT82B vps22\(^{5F3-8}\)/FRT82B cl. (C,G) eyFLP; FRT42D vps25\(^{N55}\)/FRT42D cl. (D,H) eyFLP; vps36\(^{669}\)/FRT80B/cl FRT80B. (E) eyFLP; FRT82B/FRT82B cl.

expression of \(b\kappa\kappa^{DV}\) in discs predominantly mutant for \(vps25\) does not suppress the elevated levels of \(Mmp1\) expression (Figure S3F), suggesting that other mechanisms can also induce \(Mmp1\). Thus, while inhibition of JNK signaling partially blocks apoptosis and proliferation, it has no effect on the other neoplastic characteristics seen in \(ESCRT-II\) mutant cells.

Inhibition of JAK/STAT Signaling Significantly Rescues the Neoplastic Transformation of \(ESCRT-II\) Mutant Tissues

Since we saw increased levels of JAK/STAT signaling in \(ESCRT-II\) mutant tissues, we investigated the possible autono-
Cooperation of Signaling Pathways in Tumorigenesis

|          | $ark^{H16}$ | $vps25^{N55}$, $ark^{H16}$ |
|----------|-------------|-----------------------------|
| **phalloidin** |             |                             |
| **Cas-3** |             |                             |
| **DAPI**  |             |                             |
| **BrdU**  |             |                             |
| **aPKC**  |             |                             |
| **Dig**   |             |                             |
| **phalloidin** |         |                             |
| **ELAV**  |             |                             |
| **phalloidin** |         |                             |
| **Mmp1**  |             |                             |
| **ey>bsk^{DN}** |         |                             |
| **DAPI**  |             |                             |
| **TUNEL** |             |                             |
| **phalloidin** |         |                             |
| **BrdU**  |             |                             |

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Figure 5. Inhibition of apoptosis affects the neoplastic transformation of ESCRT-II mutant tissue. Shown are predominantly mutant eye-antennal imaginal discs. Phalloidin (green) or DAPI (green) is used to mark the overall shape of the tissue. Scale bars represent 50 μm. (A–J) Apoptosis is inhibited by removal of ark (A,B); Cleaved Caspase-3 (Cas-3*; red and grayscale) labelings show that apoptosis is completely inhibited in discs predominantly mutant for vps25 and ark (B,B'). Cell death is also absent in discs predominantly mutant for ark (A,A'). (C,D) BrDU (red and grayscale) labelings show that proliferation is elevated in tissues predominantly mutant for vps25 and ark (D,D'). Proliferation is not affected in control discs predominantly mutant for ark (E–E''), (F,G) ELAV (red and grayscale) and Dlg (green and grayscale) (E,F') labelings of discs predominantly mutant for vps25 and ark show that very few cells in the double mutant discs differentiate normally (H,H'). Differentiation occurs normally in control discs predominantly mutant for ark (G,G'). (IJ) Mmp1 (red and grayscale) labelings of discs predominantly mutant for vps25 and ark show that levels of this protein are increased (I,I'). Mmp1 levels are not affected in control discs predominantly mutant for ark (I,I'). (K–N) Apoptosis is reduced by inhibition of JNK signaling by expression of the USP-bskM transgene using ey-Gal4. Phalloidin (green) or DAPI (green) is used to mark the overall shape of the tissue. (KL) TUNEL (red and grayscale) labelings show that apoptosis is reduced in discs predominantly mutant for vps25 in which JNK signaling is inhibited (L,L'). Proliferation is also low in control discs in which JNK signaling is inhibited (K,K'). (MN) BrDU (red and grayscale) labelings show that proliferation is reduced in tissues predominantly mutant for vps25 in which JNK signaling is inhibited (N,N'). Proliferation is not affected in control discs in which JNK signaling is inhibited (M,M'). Genotypes: (A,B,E,G) eyFLP/FRT42D arkD16/FRT42D cl; ey-Gal4/+; FRT42D y; FRT42D y; ey-Gal4/+; (C,D,E,G) eyFLP/FRT42D FRT42D cl; ey-Gal4/FRT42D cl; ey-Gal4/+; (L,N) eyFLP/UAS-bskM; FRT42D vps25N55 y; FRT42D vps25N55 y; ey-Gal4/FRT42D cl; ey-Gal4/+; doi:10.1371/journal.pone.0056021.g005

Loss of JAK/STAT signaling in vps22 mutant discs, however, has little to no effect on Mmp1 expression. Mmp1 levels remain elevated throughout the tissue (Figure 6H), suggesting that JAK/STAT signaling is not required for Mmp1 expression and for possible metastatic capability. Thus, elevated JAK/STAT signaling in ESCRT-II mutant tissue plays a very important role in the neoplastic transformation, leading to both disorganization of cellular architecture and failure of differentiation.

**Discussion**

While it is well established how de-regulated signaling pathways in ESCRT-II mutant clones mediate non-cell autonomous interactions with neighboring non-mutant cells to contribute to hyperplastic overgrowth and increased cell survival [1,3,5–9,13], it was largely unknown which signaling pathways trigger neoplastic transformation autonomously. To address this question, we generated predominantly mutant eye-antennal imaginal discs in which competitive interactions are eliminated so that we could examine the autonomous results of de-regulated signaling.

Overall, it appears that the same signaling pathways that are induced in mosaic clones are also activated in predominantly mutant tissues. However, two results of this study are noteworthy. First, it is surprising that JNK activity is strongly induced in tissues predominantly mutant for ESCRT-II genes. This is surprising because JNK signaling was believed to be induced by cell competition from neighboring non-mutant cells in mosaic tissues [6,16,30]. However, non-mutant tissue is largely eliminated by the ey-FLP/ey method and thus competitive interactions are eliminated. Therefore, it is not known how JNK signaling is induced in these tissues. Nevertheless, JNK signaling is critical for the overgrowth phenotype of predominantly ESCRT-II mutant eye discs as inhibition of this pathway partially blocks cell proliferation.

Second, de-regulation of the JAK/STAT signaling pathway is critical for the neoplastic transformation of vps22 mutant discs. Loss of JAK/STAT signaling dramatically normalizes the neoplastic phenotype of vps22 mutant cells. In addition to JNK and JAK/STAT activity, we also found Notch activity increased in discs predominantly mutant for ESCRT-II genes. Therefore, we tested a genetic requirement of Notch signaling for neoplastic transformation of ESCRT-II mutant cells. However, loss of Notch was inconclusive because even the wild-type control discs did not grow when Notch was inhibited (data not shown).

Interestingly, although ESCRT-II mutant tissues undergo neoplastic transformation, they also show high levels of apoptosis. Animals with predominantly mutant eye-antennal imaginal discs die as headless pharate pupae, a phenotype likely...
caused by the apoptosis of the imaginal discs before the adult stage. Reduction of JNK signaling in \textit{vps22}, \textit{vps25}, or \textit{vps36} mutant discs leads to lower levels of apoptosis, supporting a role for JNK signaling in the cell death of the predominantly mutant tissues. More excitingly, JNK also controls proliferation in these tissues, as shown by the reduction of proliferation seen when JNK signaling was down-regulated. This observation is consistent with previous findings that JNK can induce non-cell autonomous proliferation \cite{35} and that apoptosis-induced proliferation is mediated by JNK activity \cite{33,34,42}. While inhibition of JNK signaling reduces proliferation in predominantly mutant \textit{ESCRT-II} mutant discs, it does not affect other aspects of the neoplastic phenotype.

The role of JAK/STAT signaling in these mutants is complex. In mutant clones of \textit{ESCRT-II} mosaic discs, Notch-induced secretion of the JAK/STAT ligand Upd triggers non-cell autonomous proliferation \cite{35} and that apoptosis-induced proliferation is mediated by JNK activity \cite{33,34,42}. While inhibition of JNK signaling reduces proliferation in predominantly mutant \textit{ESCRT-II} mutant discs, it does not affect other aspects of the neoplastic phenotype.

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autonomous proliferation [5,7]. However, we observed that autonomous de-regulated JAK/STAT signaling in predominately mutant discs is critical for the neoplastic transformation of vps22 mutants. In vps22 Stat92E double mutant discs, organization of cellular architecture is definitively rescued with the layout of the tissue closely resembling that of a wild-type eye-antennal imaginal disc. In addition, apical-basal polarity markers are localized more-or-less correctly in these tissues, indicating that epithelial polarity is more intact. Finally, differentiation in the posterior portion of the eye disc is preserved when JAK/STAT signaling is inhibited. Thus, de-regulation of JAK/STAT signaling in vps22 mutant discs contributes to the cellular disorganization and the lack of differentiation seen in the tissues, which is consistent with a previous study that implicated JAK/STAT signaling in cell cycle control, cell size, and epithelial organization in tsg101 mutant tissues [18].

It was recently shown that cells with strong gain of JAK/STAT activity transform into supercompetitors and eliminate neighboring cells with normal JAK/STAT activity by cell competition [41]. However, in mosaic discs, a supercompetitive behavior of ESCRT-II mutant cells has not been observed. In fact, these mutant cells are eliminated by apoptosis. Only if apoptosis is blocked in these cells, is a strong overgrowth phenotype with neoplastic characteristics observed [5,6]. Thus, apoptosis can serve as a tumor suppressor mechanism to remove cells with potentially malignant JAK/STAT activity.

How endosomal trafficking specifically regulates JAK/STAT signaling and, thus, how blocking trafficking leads to increases in signaling pathway activity is interesting questions to answer in the future. It is possible that, like endocytic regulation of the Notch receptor, the endosomal pathway tightly regulates Doseless (Dome), the JAK/STAT pathway receptor. It has been shown previously that Dome is trafficked through the endocytic machinery and that this trafficking of Dome can affect the downstream output of the JAK/STAT signaling pathway [43,44]. It is also possible that Notch-induced Upd secretion causes autocrine JAK/STAT signaling in these mutants. However, technical problems (knocking down Notch function both in wild-type and mutant tissue causes general problems in tissue growth) prevented us from examining this possibility.

It will be important to examine how de-regulated JAK/STAT signaling in ESCRT-II mutants causes neoplastic transformation. JAK/STAT signaling is known to be an oncogenic pathway in Drosophila [45–49] and in humans [50] but its downstream targets that promote tumorigenesis are not yet clear. JAK/STAT signaling may be feeding into other pathways that promote tumorigenesis, such as dpp signaling [45], or may be targeting other proteins involved in transformation, such as Cyclin D [46,51–54].

A number of studies have implicated genes that function in endocytosis and endosomal protein sorting as tumor suppressors in human cancers. Most well known is Tsg101, as early studies showed that downregulation of Tsg101 promotes the growth of mouse 3T3 fibroblasts in soft agar [55]. When these cells were injected into nude mice, they formed metastatic tumors [55]. However, later studies have shown conflicting results [56–58], and it is still unclear if Tsg101 functions as a tumor suppressor in metazoans. Importantly, a number of studies have shown changes in expression of ESCRT components in human cancer cells, including changes in expression of ESCRT-I components Tsg101 [59–62] and Vps37A [63,64] and ESCRT-III components Chmp1A and CHMP3 [63,65]. Since the primary proteins that function in endocytosis and endosomal trafficking are conserved from yeast to humans, it is likely that our findings in Drosophila may have important implications for human disease.

Supporting Information

Figure S1 Notch protein levels are upregulated in vps22, vps25, and vps36 mutant tissues. Shown are predominately mutant eye-antennal imaginal discs. Scale bars represent 50 μm. Phalloidin (green) is used to mark the overall shape of the tissue. Notch protein levels (red, grayscale) are shown by staining with an antibody recognizing the intracellular domain of the protein (α-Nintr). Notch protein levels are increased in imaginal discs predominantly mutant for vps22 (B), vps25 (C), or vps36 (D), as compared to Notch protein levels in control discs (A). Genotypes: (A) eyFLP; FRT82B/FRT82B cl. (B) eyFLP; FRT82B vps22k55 m/FRT82B cl. (C) eyFLP; FRT42D vps25k55 y'/FRT42D cl. (D) eyFLP; vps36Δ4690 FRT80B/el FRT80B. (TIF)

Figure S2 JNK signaling is upregulated in tissues predominantly mutant for vps25. Shown are predominately mutant eye-antennal imaginal discs. Phalloidin (green) is used to mark the overall shape of the tissue. puc-lacZ is detected by β-gal labeling (red or grayscale). (A-B) Imaginal discs predominantly mutant for vps25 induce high levels of puc-lacZ (B), as compared to puc-lacZ expression in control discs (A). Scale bars represent 50 μm. Genotypes: (A) eyFLP; FRT42D/FRT42D cl; puc-LacZ+/+. (B) eyFLP; FRT42D vps25k55 y'/FRT42D cl; puc-LacZ+/+. (TIF)

Figure S3 Inhibition of JNK signaling does not affect the disorganization of cellular architecture, the failure of differentiation, and the invasive potential of ESCRT-II mutant tissue. Shown are predominantly mutant eye-antennal imaginal discs. JNK signaling is inhibited by expression of the UAS-brok transgene using ey-Gal4. Phalloidin (green) is used to mark the overall shape of the tissue. Scale bars represent 50 μm. (A,B) αPKC (red and grayscale (A',B')) and Dlg (green and grayscale (A'',B'')) labelings of discs predominantly mutant for vps25 in which JNK signaling is inhibited show that cellular architecture is disrupted (B''). Cellular architecture is not disrupted in control discs in which JNK signaling is inhibited (A''). Genotypes: (A) eyFLP; FRT42D/FRT42D cl; puc-LacZ+/+. (B) eyFLP; FRT42D vps25k55 y'/FRT42D cl; puc-LacZ+/+. (C) eyFLP; eyFLP; puc-lacZ m. Phalloidin (green) is used to mark the overall shape of the tissue. Scale bars represent 50 μm. Genotypes: (A) eyFLP; FRT42D/FRT42D cl; puc-LacZ+/+. (B) eyFLP; FRT42D vps25k55 y'/FRT42D cl; puc-LacZ+/+. (TIF)

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Author Contributions
Conceived and designed the experiments: SEW AB. Performed the experiments: SEW HKG JAH. Analyzed the data: SEW HKG AB. Contributed reagents/materials/analysis tools: SEW HKG JAH AB. Wrote the paper: SEW AB.

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