ESCRTs: from Cell Biology to Pathogenesis


The role of ESCRT proteins in attenuation of cell signalling

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Abstract
The ESCRT (endosomal sorting complex required for transport) machinery consists of four protein complexes that mediate sorting of ubiquitinated membrane proteins into the intraluminal vesicles of multivesicular endosomes, thereby targeting them for degradation in lysosomes. In the present paper, we review how ESCRT-mediated receptor down-regulation affects signalling downstream of Notch and growth factor receptors, and how ESCRTs may control cell proliferation, survival and cytoskeletal functions and contribute to tumour suppression.

Introduction

The ESCRT (endosomal sorting complex required for transport) machinery is essential for proper down-regulation of signalling receptors [1]. It was originally identified as three protein complexes (ESCRT-I, -II and -III) that mediate sorting of ubiquitinated membrane proteins to the lumen of the lysosome-like vacuole in the yeast Saccharomyces cerevisiae [2]. The Vps (vacuolar protein sorting) 27-Hse1 (has symptoms of class E mutants 1) complex [Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate)–STAM (signal-transducing adaptor molecule) in mammals], which functions upstream of ESCRT-I [3–5], is often regarded as a fourth ESCRT, and here we will refer to this complex as ‘ESCRT-0’. Yeast mutants with defective ESCRT functions not only display sorting defects, but also fail to accumulate ILVs (intraluminal vesicles) within endosomes and vacuoles, indicating that ESCRTs play a central role in both endosomal receptor sorting and biogenesis of multivesicular endosomes [2].

All four ESCRTs are conserved from yeast to humans, although some organisms, including plants, lack ESCRT-0 [6]. Although originally described for its involvement in degradative protein trafficking, accumulating evidence suggests that the ESCRT machinery also represents a conserved pathway for spatial and temporal restriction of receptor signalling [1,7]. Receptors activated by ligand at the plasma membrane, including Notch and RTKs (receptor tyrosine kinases), become ubiquitinated and endocytosed. In the endosome membrane, the ubiquitinated receptors are recognized by the ESCRT machinery, which serves to sort the receptors into ILVs, thereby targeting them for degradation in lysosomes and terminating their signalling [6,8,9].

The subunit composition and biochemistry of the ESCRTs have been reviewed extensively elsewhere [6,9] and will not be described in detail here. In brief, ESCRT-0 is recruited to endosome membranes through binding of the Vps27/Hrs subunit to PtdIns3P [10], whereas ESCRT-I is recruited through an interaction between its Vps23/Tsg101 (tumour susceptibility gene 101) subunit and the Vps27/Hrs subunit of ESCRT-0 [3–5]. ESCRT-II interacts with both ESCRT-I and PtdIns3P [11,12], and ESCRT-III assembles as a polymeric complex on membranes, aided by the interaction of its Vps20 subunit with ESCRT-II [13,14]. Whereas ESCRT-0, -I and -II contain...
ubiquitin-binding subunits thought to interact sequentially with ubiquitinated cargo, ESCRT-III recruits deubiquitinating enzymes that presumably serve to deubiquitinate cargo before its inclusion in ILVs [15]. ESCRT-III also recruits an AAA (ATPase associated with various cellular activities), Vps4, which serves to disassemble ESCRT-III [13,16,17]. Even though the ESCRTs are thought to function consecutively in sorting of ubiquitinated membrane proteins into ILVs, the exact mechanisms of sorting and ILV formation, and the relationships between these events, are not known [6,9].

The importance of ESCRTs in receptor down-regulation is highlighted by the neoplastic growth of Drosophila melanogaster tissues that lack ESCRT subunits [18]. In the present paper, we review recent studies that shed light on signalling pathways that are controlled by ESCRTs and discuss how their dysregulation may cause tumorigenesis.

### ESCRTs and RTK signalling

The importance of ESCRTs for attenuation of receptor signalling has been studied in cell culture systems using RNAi (RNA interference) or mouse, Drosophila and Caenorhabditis elegans mutants. RNAi knockdown experiments in mammalian cell lines have shown that depletion of Hrs (ESCRT-0) or Tsg101 (ESCRT-I) recruits deubiquitinating enzymes that presumably serve to deubiquitinate cargo before its inclusion in ILVs [15]. ESCRT-III also recruits an AAA (ATPase associated with various cellular activities), Vps4, which serves to disassemble ESCRT-III [13,16,17]. Even though the ESCRTs are thought to function consecutively in sorting of ubiquitinated membrane proteins into ILVs, the exact mechanisms of sorting and ILV formation, and the relationships between these events, are not known [6,9].

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The use of multicellular model organisms, particularly Drosophila, has contributed significantly to shed light on the role of ESCRTs in modulation of cell signalling (Table 1). Consistent with the results from cell culture, hrs mutant Drosophila larvae show increased levels of tyrosine-phosphorylated EGFRs and activated MAPK [25,26]. Another RTK, Torso, is also activated in hrs mutants [25], and, because most RTKs are thought to be down-regulated in a ubiquitin-dependent manner, multiple RTK pathways are likely to be affected by inactivation of ESCRT subunits.

### ESCRTs and Notch signalling

The transmembrane receptor Notch is a major regulator of cell proliferation and differentiation. Both Notch and its transmembrane ligands undergo ubiquitination, and the ubiquitin-dependent intracellular trafficking of these molecules profoundly affects their signalling [27]. Proteolytic activation and signalling of Notch is reduced when entry into the early endosome is inhibited, but is enhanced when ESCRT function is abolished [28]. Notch receptor and its transmembrane ligand Delta accumulate in the endosomes of ESCRT mutant cells, and the increased signalling observed is presumably

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**Table 1** | Signalling pathways activated or up-regulated when ESCRT function is inhibited

<table>
<thead>
<tr>
<th>Subunit</th>
<th>ESCRT</th>
<th>Model</th>
<th>Organism or cell line</th>
<th>Pathway or component activated</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hrs 0 mut</td>
<td>Dm</td>
<td>EGFR</td>
<td></td>
<td></td>
<td>[25]</td>
</tr>
<tr>
<td>Hrs 0 mut, KD</td>
<td>Dm, Hs HeLa</td>
<td>ERK1/2</td>
<td></td>
<td></td>
<td>[23,25]</td>
</tr>
<tr>
<td>Hrs 0 KD</td>
<td>Hs HeLa</td>
<td>Met (HGF receptor)</td>
<td></td>
<td></td>
<td>[52]</td>
</tr>
<tr>
<td>Hrs 0 KO</td>
<td>Mm</td>
<td>Apoptosis</td>
<td></td>
<td></td>
<td>[32]</td>
</tr>
<tr>
<td>Tsg101 I mut</td>
<td>Dm</td>
<td>Hid</td>
<td></td>
<td></td>
<td>[35]</td>
</tr>
<tr>
<td>Tsg101 I KD</td>
<td>Mm 3T3, Hs HeLa</td>
<td>Notch, JAK/STAT (non-autonomous)</td>
<td></td>
<td></td>
<td>[30]</td>
</tr>
<tr>
<td>Tsg101 I KO</td>
<td>Mm</td>
<td>p53, p21cip1</td>
<td></td>
<td></td>
<td>[33]</td>
</tr>
<tr>
<td>Tsg101 I mut</td>
<td>Dm</td>
<td>Proliferation</td>
<td></td>
<td></td>
<td>[30]</td>
</tr>
<tr>
<td>Tsg101 I KO</td>
<td>Mm</td>
<td>Apoptosis</td>
<td></td>
<td></td>
<td>[34]</td>
</tr>
<tr>
<td>Vps28 I mut</td>
<td>Dm</td>
<td>Actin remodelling</td>
<td></td>
<td></td>
<td>[38]</td>
</tr>
<tr>
<td>Vps25 II mut</td>
<td>Dm</td>
<td>Dpp (TGFβ), Tkv (TGFβ receptor)</td>
<td></td>
<td></td>
<td>[26,29]</td>
</tr>
<tr>
<td>Vps25 II mut</td>
<td>Dm</td>
<td>Hippo</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>Dm</td>
<td>JNK</td>
<td></td>
<td></td>
<td>[35]</td>
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<tr>
<td>Vps25 II mut</td>
<td>Dm</td>
<td>Hid</td>
<td></td>
<td></td>
<td>[35]</td>
</tr>
<tr>
<td>Vps25 II mut</td>
<td>Dm</td>
<td>Proliferation</td>
<td></td>
<td></td>
<td>[29,31]</td>
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<tr>
<td>Vps25 II mut</td>
<td>Dm</td>
<td>Apoptosis</td>
<td></td>
<td></td>
<td>[29,31]</td>
</tr>
</tbody>
</table>
related to the increased residence time of the receptor–ligand complex in the limiting membrane of the endosomes [29]. One important consequence of activation of the Notch signalling pathway in *Drosophila* ESCRT mutants is increased transcription of the cytokine Upd (Unpaired), which becomes secreted by ESCRT mutant cells. Upd binds receptors on adjacent cells and acts as a mitogen by activating a JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signalling pathway, thereby causing cell proliferation in a non-autonomous manner [29–31] (see below).

**ESCRTs and apoptosis**

Both ESCRT-0 and ESCRT-I are required for embryonic development and viability, as demonstrated by the early embryonic lethality of mice lacking Hrs or Tsg101 [32,33]. Tsg101 mutant mouse embryos, which die at embryonic day 6.5, show a decrease in cellular proliferation *in vivo* and *in vitro* correlating with down-regulation of the cyclin-dependent kinase CDK2, an essential factor for S-phase entry of the cell cycle [33]. Although no apoptosis was observed in the initial study of Tsg101-knockout embryos, later studies with conditional knockout of Tsg101 in mouse mammary epithelium showed pronounced apoptosis of differentiated cells [34]. Likewise, hrs mutant embryos, which die around embryonic day 11, exhibit significant apoptosis within the endoderm of the ventral region [32]. A strong activation of apoptosis is also detected in *Drosophila* tissues that are mutant for the ESCRT-II subunit Vps25 [29,31,35]. This effect is correlated with sensitivity to cell competition [29] and could be related to the inability of the mutant cells to exit the cell cycle [31]. Several pro-apoptotic molecules are activated in *vps25* mutant *Drosophila* clones, including Hippo (corresponding to Mst1/2 in mammals), JNK (c-Jun N-terminal kinase) and Hid, and there is also a down-regulation of the caspase inhibitor Diap1 (corresponding to IAP1 inhibitor of apoptosis protein 1) in mammals, which is antagonized by Hid [35]. Surprisingly, however, Diap1 is strongly up-regulated in wild-type cells adjacent to *vps25* mutant cells, suggesting that *vps25* mutant cells may promote non-autonomous cell survival. Indeed, enlarged eye imaginal discs arising from expression of mutant clones of Vps25 consist almost entirely of wild-type tissue, with very low content of remaining mutant cells [18].

An additional interface between ESCRTs and apoptosis might be provided by Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X], a cytosolic protein originally identified as a binding partner for the pro-apoptotic regulator ALG-2 [36]. Up-regulation of Alix correlates with increased apoptosis, suggesting that Alix might function together with ALG-2 in apoptosis induction. Of possible relevance for the apoptosis phenotype of ESCRT mutants, Alix interacts with Tsg101 in ESCRT-I and with Vps32/CHMP4 (charged multivesicular body protein 4) in ESCRT-III, and this interaction appears to be crucial for the ability of Alix to promote neuronal apoptosis [37]. However, it remains to be clarified whether ESCRT depletion affects the pro-apoptotic activity of Alix.

**ESCRTs and the actin cytoskeleton**

An unexpected observation in *Drosophila* mutants with impaired ESCRT-I function is the profound effects on the actin cytoskeleton, as demonstrated in embryos lacking the ESCRT-I subunit Vps28. Several processes that require the actin cytoskeleton are perturbed in *vps28* mutants, including axial migration of nuclei, formation of transient furrows during cortical divisions in syncytial embryos and the subsequent cellularization [38]. One proposed mechanism to explain the effects of Vps28 disruption on the actin cytoskeleton is a failure to recruit the dystrophin-like Dah protein to invaginating furrows during cell division. This is a plausible explanation because Dah is thought to anchor actin filaments to membranes, although the mechanisms that link Dah recruitment to Vps28 function remain obscure. Since Dah cannot account for all the effects of Vps28 deficiency on actin organization [38], additional mechanisms must also be sought.

Except for Vps28, the importance of ESCRTs for cytoskeletal organization has not been investigated much so far, although such a relationship could be important for understanding the tumour phenotypes of ESCRT mutants (see below). A possible link between ESCRTs and the actin cytoskeleton is the ESCRT-I- and -III-binding protein Alix (see above). Depletion of Alix causes redistribution of early endosomes and accumulation of unusual actin structures that contain clathrin and cortactin, a protein that couples membrane dynamics to the cortical actin cytoskeleton [39]. Other possible connections between the ESCRT machinery and the actin cytoskeleton are provided by the ESCRT-0 subunit Hrs. Hrs interacts with merlin, which is involved in linking the actin cytoskeleton to the plasma membrane [40], and Hrs can also be found in a complex that contains actinin-4 and myosin V [41]. However, the functional consequences of Hrs depletion on cytoskeletal dynamics have not been clarified.

**ESCRT subunits as tumour suppressors**

The ESCRT-I subunit Tsg101 was originally identified as a tumour suppressor in mice, based on the finding that implantation of cells with reduced Tsg101 expression caused tumours in nude mice [42]. Paradoxically, however, subsequent experiments with conditional Tsg101-knockout mice have failed to show any tumour-suppressor phenotypes [34]. On the contrary, mice overexpressing Tsg101 contain increased activation of the EGFR pathway, and, in these mice, Tsg101 has a weak oncogenic effect [43]. Likewise, although Tsg101 was originally reported to be down-regulated in cancers, Tsg101 is actually up-regulated in some cancers [43]. The view of Tsg101 as a tumour-suppressor mechanism in mammals therefore remains highly controversial (reviewed in [44]). However, it is worth noting that also another ESCRT-I subunit, Vps37, which has four isoforms in humans, has been implicated in tumour suppression. Depletion of one of these isoforms, HCRP1 (hepatocellular carcinoma-related protein 1)/Vps37A [45], enhances proliferation and elevates the invasive ability of hepatocellular carcinoma cells [46].
Further studies are needed in order to establish whether Tsg101, Vps37A and other subunits can function as *bona fide* tumour suppressors in humans.

To date, the most compelling evidence for the concept that ESCRT subunits can function as tumour suppressors comes from the fact that the ESCRT-I, -II and -III components Tsg101, Vps25 and Vps32 have been identified in screens for tumour suppressors in *Drosophila* [29–31,47]. The expression of clones of ESCRT mutant cells in *Drosophila* imaginal discs causes neoplastic growth of the mutant cells which is due to overproliferation and loss of epithelial organization. In addition, there is overproliferation and hyperplasia of neighbouring wild-type tissue. This non-autonomous tumorigenic effect caused by lack of ESCRT function has been attributed to the secretion of the mitogen Upd from the mutant cells, triggered by activation of the Notch pathway. It is important to note, however, that down-regulation of STAT92E, which transduces the extracellular Upd signal to the nucleus of receiving cells, causes a less complete suppression of vps25 phenotypes than of overproliferation mediated by Upd alone [31]. This suggests that additional factors contribute to vps25-induced tissue overgrowth. Possible candidates are Wingless and Dpp (a transforming growth factor-β like cytokine), which are secreted by ESCRT mutant cells [29,30].

Whereas unrestricted proliferation and loss of polarity are key events in oncogenesis, the development of malignant metastatic tumours also involves invasion of cancer cells into adjacent or remote tissue. An interesting transplantation experiment suggests that ESCRT mutant cells may be capable of such invasion in *Drosophila* if their apoptosis is inhibited. When portions of eye imaginal discs containing clones of vps25 mutant cells expressing an apoptosis inhibitor were transplanted into the abdomens of adult fruitflies, two out of 15 animals showed invasion of these cells to other parts of the fly [29]. The molecular mechanisms underlying this invasion phenotype are not known, and it remains to be investigated whether ESCRT mutant cells can invade other tissues in the absence of apoptosis inhibitors.

A surprising aspect of the tumour phenotypes caused by ESCRT deficiency in *Drosophila* is that these are found in ESCRT-I, -II and -III mutants, but not in ESCRT-0 mutants [35,47]. Taking into account that ESCRT-0 functions upstream of ESCRT-I, how can this be explained? One clue may come from the finding that hrs mutants, unlike vps25 and tsg101 mutants, show no ectopic activation of Notch signalling even though there is a strong accumulation of Notch in endosomes [29,31]. Since Notch is likely to be trapped at a slightly earlier point during endosomal maturation in hrs mutants than in tsg101 and vps25 mutants, it is possible that activation of Notch signalling from endosomes requires an activation event that occurs downstream of Hrs, but upstream of Tsg101. Alternatively, Hrs, which is known to interact with a large number of molecules [48], might control Notch signalling by mechanisms that are independent of the ESCRT pathway, thereby perhaps eliciting compensatory silencing of Notch signalling when Hrs is inactivated. Perhaps relevant to this, Hrs has been reported to engage in ESCRT-independent complexes that mediate receptor recycling rather than lysosomal sorting [41,49].

In conclusion, the neoplastic phenotype observed in ESCRT mutant *Drosophila* is mainly due to overproliferation and loss of epithelial organization, and the hyperplastic phenotype is caused by a non-autonomous stimulation of cell proliferation, possibly in conjunction with a non-autonomous inhibition of apoptosis (Figure 1). Apoptosis of ESCRT mutant cells causes these to become nearly undetectable in some tumours and counteracts the neoplastic phenotype [31]. Interestingly, when the apoptotic phenotype is abolished by expression of the caspase inhibitor p35 in the ESCRT mutant cells, a spectacular overgrowth of *Drosophila* imaginal discs can be observed, indicating that the mutant cells receive strong signals for proliferation that are masked by the apoptosis induction [29,30]. The sources of these autonomous proliferative signals are not known, but RTKs such as the EGFR are good candidates. Autonomous Notch signalling is also likely to contribute to the proliferative signal of ESCRT mutant cells, and it is possible that these cells contain autocrine signalling loops via secreted ligands such as Upd, Dpp and Wnt [29].

The proliferative signalling found in *Drosophila* mutant cells contrasts with the antiproliferative signals observed in conditional Tsg101-knockout mice (down-regulation of CDK2 and up-regulation of p53 and p21<sup>WAF1</sup> [33]) and might provide some of the explanation as to why only the former form tumours. It must also be taken into account that the most prominent tumorigenesis in *Drosophila* ESCRT mutants occurs in the presence of apoptosis inhibitors. Perhaps the complex balance between autonomous apoptosis and non-autonomous proliferation signals might explain the differences found between mouse mammary epithelium and fruitfly imaginal discs with respect to the tumour-suppressor activities of ESCRT components. In the Tsg101-knockout mice, a whole tissue is devoid of Tsg101, whereas, in the Drosophila mutants, only clones of cells have the mutant phenotype, a situation not unlike development of human cancers. It might thus be interesting to generate mice with clonal inactivation of ESCRT subunits in various epithelia in order to mimic these conditions.

### Conclusion and perspectives

By controlling the residence time of endocytosed signalling receptors in endosomes, the ESCRTs regulate signalling output from these organelles. Although it is evident that Notch and RTK signalling is up-regulated in the absence of proper ESCRT functions, we know little about most other signalling pathways so far. Because multiple receptors are known to become ubiquitinated upon ligand stimulation [50,51], the ESCRTs are likely to control trafficking and signalling of diverse receptor signalling pathways.

With respect to ESCRTs and cell signalling, several key questions remain to be addressed. Are ESCRT subunits other than Vps28 involved in control of the actin cytoskeleton, and what are the mechanisms? How do ESCRTs control epithelial cell polarity? How does ESCRT ablation promote tumour
How inhibition of ESCRT function may promote tumorigenesis in Drosophila mosaic epithelia

In ESCRT mutant cells, aberrant signalling occurs from aberrant endosomes, including RTK and Notch signalling as well as signalling from a number of unknown receptors (indicated with question marks). The net result of such signalling is stimulation of proliferation and loss of polarity, both of which are prerequisites for neoplastic growth. Stimulation of actin remodelling possibly contributes to this phenotype. Acting against these cell-autonomous neoplastic stimuli is the activation of apoptosis, which promotes death of ESCRT mutant cells. Mutant cells that escape apoptotic death may form neoplastic tumours. Notch activation occurring in the ESCRT mutant cell also results in secretion of the cytokine Upr, which can activate Hrs cytokine receptors in neighbouring wild-type cells, thereby stimulating their proliferation in a non-autonomous manner. An unknown signal also stimulates these cells to up-regulate the caspase inhibitor Diap1 (an E3 ubiquitin ligase that polyubiquitinates the initiator caspase Dronc and thereby mediates its degradation in proteasomes), thus promoting cell survival. The combined stimulation of proliferation and survival cause hyperplastic growth of the wild-type cells surrounding the ESCRT mutant cells. Solid arrows indicate connections for which experimental evidence exists, whereas broken arrows indicate hypothetical connections.

References


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