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GENETIC SCREENING TO IDENTIFY INTERACTORS OF ESCRT-II SUBUNIT, VPS25, AND PRELIMINARY CHARACTERISATION OF CANDIDATES

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4 ABSTRACT

ESCRT (Endosomal Sorting Complex Required for Transport) proteins regulate cell surface receptor degradation by sorting and packaging ubiquitinated cargoes into the intraluminal vesicles of multivesicular bodies (MVBs). A range of human diseases including cancer, and neurodegeneration display altered expression or are caused by mutations of ESCRT subunits. Studies have shown that Drosophila tissues lacking ESCRTs display neoplastic-like features like overproliferation and polarity defects, partly due to aberrant signalling including Notch signalling. To understand ESCRT-regulated processes in vivo, we utilised modification of a deformed wing phenotype specifically caused by knockdown (RNAi) of Vps25, an ESCRT-II subunit. We systematically screened chromosomal regions and identified 204 genetic interactors of Vps25 that enhanced/suppressed the phenotype. They include genes that function in trafficking, signalling, transcription, ion transport and many other biological processes; suggesting that ESCRTs influence a wide range of biological processes. We have focused on a subset of these hits that regulate tissue growth with a secondary screen based on modification of a Delta-driven eye overgrowth phenotype, isolating a subset of 43 genes involved in regulating tissue growth, some of which are novel and uncharacterised. Human orthologues of some of these genes are important in cancers; dropout (dop), whose mammalian orthologues are the MAST kinases, have been shown to contribute towards breast cancer development. dop mediates Delta-driven eye overgrowth possibly by upregulating Delta expression. In human cells, MAST2 does not affect Notch signalling but might contribute to tumorigenesis by regulating the NFkB pathway. We have also characterised another interactor, CG12163 which is the homologue of mammalian Cathepsin F. Mutations in Cathepsin F cause a rare form of neuronal ceroid lipofuscinosis (NCL) called Type B Kufs disease. Our Drosophila model which recapitulates aspects of the human disease phenotype suggests that defects in autophagy might underlie the pathogenesis of NCLs.
5 INTRODUCTION

One of the defining features of eukaryotic cells is compartmentalization, that is, the formation of cellular organelles surrounded by membranes. It is thought to have propelled life towards multi-cellularity and emergence of a nervous system (Cavalier-Smith, 2002; Dacks and Field, 2007). Most of the cellular logistics, which involves incessant trafficking of countless cargoes and associated macromolecules, are enabled by the plasma membrane and endo-membrane system. This process is crucial for cell fate and identity, as well as cell communication.

5.1 Discovery and organisation of the ESCRT machinery

The ESCRT machinery was first identified in yeast by genetic isolation of mutants that cause defective protein sorting to the vacuole, the functional equivalent of the lysosome (Bankaitis et al., 1986; Rothman et al., 1989). These mutants possessed enlarged pre-vacuolar endosome-like compartments containing undegraded proteins, and were called ‘class E-vps mutants’ (Raymond et al., 1992). Most of the class E-vps genes were later found to act in succession to concentrate trafficking cargoes and include them into forming late endosomes (also termed multivesicular bodies or MVBs) that eventually fuse with lysosomes for degradation (Katzmann et al., 2001). The ESCRT machinery that regulates endosomal sorting is organized into five distinct protein complexes: ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and the Vps4 AAA-ATPase complex (see Table 1 for subunit compositions).
Table 1: Composition of the ESCRT complexes in endosomal sorting

<table>
<thead>
<tr>
<th>Complex</th>
<th>Function</th>
<th>Evolutionary origin</th>
<th>Yeast</th>
<th>Drosophila</th>
<th>Human</th>
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<tr>
<td>ESCRT-0</td>
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<td>Vps27</td>
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<td>Vps25</td>
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<td>Vps2</td>
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<td>CG7967</td>
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**Figure 1: The ESCRTs in MVB biogenesis and receptor sorting.** ESCRT-0, -I and -II interact with ubiquitinated cargo through their ubiquitin-binding domains, and sort them into intraluminal vesicles (ILVs). ESCRT-II recruits ESCRT-III to pinch/sever the ILV from the limiting MVB membrane. ESCRT-III polymeric filaments are responsible for the ILV abscission. The receptor is deubiquitinated before ILV formation. The Vps4 ATPase complex then disassembles the ESCRT-III complex for subsequent rounds of sorting. *Adapted from* (Rusten et al., 2012)

**5.2 Mechanism of ESCRT function in endosomal sorting**

During sorting, the ESCRT complexes are recruited sequentially from the cytoplasm to the endosomal membrane by interaction of specific subunits (Figure 1). Ubiquitination of cargoes provides the key signal for initial cargo binding by ESCRT-0 (see for review (Urbé, 2005)). Indeed, the ESCRT-0 subunits Hrs and Stam, as well as the ESCRT-I Vps23/Tsg101 and the ESCRT-II Vps36, all contain ubiquitin-binding domains that interact with ubiquitinated cargoes. ESCRT-0 is also recruited by interaction between the FYVE domain of Hrs and phosphatidylinositol 3-phosphate (PI3P), which is enriched at the endosomal membrane. ESCRT-0 is thought to concentrate ubiquitinated cargoes by organizing flat coats of clathrin
on the endosomal membrane (Raiborg et al., 2001, 2002). ESCRT-0 also summons ESCRT-I that retains the cargoes by ubiquitin binding and hands them to the ESCRT–II complex. The ESCRT-II complex provides a scaffold for the formation of the ESCRT-III complex, the business end of the ESCRT machinery. The Vps32/Snf7/Chmp4 subunit of ESCRT-III forms multimeric filaments organized in spirals that bend the endosomal membrane away from the cytoplasm to form invaginated buds. Thus, the combined activity of ESCRTs allows sorted cargoes to be corralled and trapped in nascent intraluminal vesicles (ILVs) of the MVBs that eventually pinch off into the endosomal lumen. The deubiquitinating enzyme Doa4 is recruited by ESCRT-III to remove ubiquitin from cargoes that are included into ILVs. Finally, the Vps4 ATPase complex binds and fully unfolds the ESCRT-III complex in an ATP-dependent manner and favors pinching off the ILV neck, the final step of MVB biogenesis (Adell et al., 2014; Bache et al., 2003; Henne et al., 2012; Mageswaran et al., 2014; Malerød and Stenmark, 2009; Shields et al., 2009; Teis et al., 2010; Wollert and Hurley, 2010; Yang et al., 2015). The structure of most ESCRT components has been determined and detailed extensive knowledge of the ESCRT mechanism of action in endosomal sorting and MVB biogenesis is available (see for review (Hurley, 2010; Williams and Urbé, 2007)).

5.3 ESCRTs activity during trafficking processes distinct from endosomal sorting

The ESCRT-I, II, III and the Vps4 complexes are conserved across the eukaryotic lineage (Wideman et al., 2014). In contrast, the ESCRT-0 is present only in subset of eukaryotes. This indicated early on that they are specialized to couple the core membrane-remodeling activity of ESCRT-III and Vps4 with cargo sorting. Indeed, evidence indicates that additional complexes, such as those containing the protein Tom1, might control initial concentration of ubiquitinated cargoes in endosomes (Blanc et al., 2009). Consistent with the accessory role of
ESCRT-0, a large body of studies in the last 25 years revealed that the function of the ESCRTs at membranes is not limited to endosomal sorting and MVB biogenesis (Figure 2).

**Figure 2: Summary of ESCRT-dependent processes.** In addition to the well-known role of ESCRTs in receptor sorting and MVB biogenesis, several processes have been shown to require the function of ESCRT proteins. Specific factors required for each process are written in italics. (Alfred and Vaccari, 2016).
5.3.1 ESCRTs and membrane budding

Early work indicated that a number of viruses can recruit ESCRT-III and Vps4 to bud from the plasma membrane (Garrus et al., 2001; Pornillos et al., 2002), leading to subsequent realization that budding of the plasma membrane operated by ESCRTs occurs also in non-infected cells to form ectovesicles. MVBs can also fuse to the plasma membrane to release of ILVs, in this case referred to as exosomes. As in endosomal sorting, deployment of ESCRT-III and Vps4 in exosome and ectovesicle (together referred to as exovesicles) formation appears to depend either on the ESCRT-I and –II, or on Alix, and to require adapters different from the ESCRT-0. These data indicated that MVB and exovesicle biogenesis can profoundly differ and that multiple pathways of exovesicle formation are likely to exist (Baietti et al., 2012; Choudhuri et al., 2014; Colombo et al., 2013; MacDonald et al., 2015; Nabhan et al., 2012; Wehman et al., 2011).

5.3.2 ESCRTs and autophagy

MVBs also act as main stations for autophagic trafficking (Kaur and Debnath, 2015; Lamb et al., 2013). Evidences from Caenorhabditis elegans, Drosophila and mammalian cell in culture revealed that ESCRTs are required both for micro-autophagy and macro-autophagy (Morelli et al., 2014; Roudier et al., 2005; Rusten et al., 2007; Sahu et al., 2011). During macroautophagy, autophagosomes that are formed de novo to clear long-lived proteins, cytoplasmic aggregates or damaged organelles fuse to MVBs and lysosomes to form amphisomes and autolysosomes respectively, in which content is progressively degraded. While it recently emerged that ESCRT activity is coordinated with macroautophagic response to starvation (Jones et al., 2012; Müller et al., 2015), how ESCRT regulate autophagy mechanistically is currently unclear. In summary, the membrane trafficking functions regulated by ESCRTs are crucial for lysosome-mediated cargo degradation, for release of exovesicles and, perhaps indirectly, for autophagy.
5.4 Non-trafficking functions of ESCRTs

5.4.1 ESCRTs and cytokinesis

The first evidence of ESCRT functions that are independent of membrane trafficking indicated that ESCRT-III and Vps4 act at the plasma membrane to sever microtubules and release the midbody during cytokinesis. In this case, the recruitment is operated by the midbody protein Cep55, with ESCRT-III directly recruiting the microtubule severing protein Spastin. This activity is present also in Archea and plants, suggesting that it is ancient evolutionarily (Carlton and Martin-Serrano, 2007; Lindås et al., 2008; Samson and Bell, 2009). Very recent studies showed similar recruitment of Spastin during nuclear envelope reformation at the end of mitosis, albeit with a different recruitment system (Olmos et al., 2015; Vietri et al., 2015).

5.4.2 ESCRTs function at the membrane

The roles of ESCRTs at the nuclear membrane began to emerge with the recognition that ESCRTs are required for budding of the Epstein–Barr virus through the nuclear membrane (Lee et al., 2012). More recently, it was found the ESCRT machinery also restores membrane integrity upon nuclear pore and nuclear envelope damage (Olmos et al., 2015; Raab et al., 2016; Webster et al., 2014). These membrane repair functions of the ESCRT machinery are also observed at the plasma membrane (Jimenez et al., 2014) and a likely developmental counterpart of such activity has been observed in neuron remodeling. Indeed, ESCRTs have been shown to be required for membrane scission that occurs during neuron pruning (Loncle et al., 2015). ESCRT-dependent neuronal remodeling events were described previously in Drosophila development but had been attributed to endo-lysosomal trafficking of neuronal receptors (Issman-Zecharya and Schuldiner, 2014; Sweeney et al., 2006; Zhang et al., 2014).

5.4.3 Less studied functions of ESCRTs

Other less understood ESCRT functions include control of centrosome number during mitosis (Frost et al., 2012; Jin et al., 2005; Morita et al., 2010; Xie et al., 1998), transcriptional gene
regulation (Burgdorf et al., 2004; Kamura et al., 2001; Lin et al., 2013; Schmidt et al., 1999; Stauffer et al., 2001; Sun et al., 1999), RNA transport (Irion and St Johnston, 2007), and miRNA biogenesis (Gibbings et al., 2009; Lee et al., 2009b). The mechanistic details of these processes are unclear. While it is reasonable to think that bending of membranes away from the cytoplasm might be involved - which to date represents the shared topological feature of all well-characterized processes operated by ESCRT - ESCRTs could also possess moonlighting functions that do not involve membranes.

5.5 ESCRTs, signalling and tissue architecture

Because a number of signalling proteins are transmembrane or membrane-associated, endocytosis and trafficking to lysosomes are crucial to regulate signal transduction (see for review (Sigismund et al., 2012)). Indeed, studies in cells of multicellular organisms that followed the initial discovery of ESCRT in yeast revealed that endosomal sorting complexes are essential to downregulate signalling, most prominently Epidermal Growth Factor (EGF)-stimulated signalling (Babst et al., 2000; Bache et al., 2004, 2006; Baldys and Raymond, 2009; Bishop et al., 2002; Chanut-Delalande et al., 2010; Doyotte et al., 2005; Jékely and Rørth, 2003; Lloyd et al., 2002).

5.5.1 ESCRTs and Notch signalling

5.5.1.1 Notch signalling

An additional signalling pathway that is well known to be regulated by ESCRTs is that controlled by Notch receptors. Core components of the Notch pathway take part in so-called canonical signalling events initiated by interaction of Delta/Serrate/Lag2 (DSL) ligands - expressed by signal-sending cells - with the Notch receptor at the plasma membrane of receiving cells. Canonical signalling requires emergence to the plasma membrane of Notch as a heavily glycosylated heterodimer. Such modifications and cleavage operated by the serine
protease Furin (also called S1 cleavage) occur in the Golgi apparatus. Binding of DSL ligands in trans is thought to displace the extracellular domain of Notch, which is held in place by Ca$^{2+}$ interactions. Shedding of extracellular Notch requires endocytosis of the ligand in the signal-sending cell. It has been proposed that the Notch receptor is deformed by the pulling forces of the endocytosis in such a way to reveal a site for cleavage by metalloproteases (called S2 cleavage). Metalloproteases cleavage turns Notch into a substrate for a final cleavage (S3 cleavage) by the $\gamma$-secretase complex on the cytoplasmic side of the plasma membrane (Figure 3).

As Notch molecules are constitutively targeted to lysosomes for ubiquitin-dependent degradation, cleavage events during canonical signalling are likely to occur also on the endosomal membrane. In all cases of canonical signalling, once the intracellular domain of Notch (NICD) is liberated from membranes, it accesses the nucleus where it regulates transcription of target genes by de-repressing the CBF1/Su(H)/Lag-1 (CSL) [RBP-Jκ in mammals, Su(H) in Drosophila] transcription complex (Figure 3). Signal termination is ensured by ubiquitin-dependent proteasomal degradation of NICD [see for review (Bray, 2016)].
Figure 3: Canonical Notch Signalling Pathway. (A) Notch is cleaved to NICD by γ-secretase at the plasma membrane, or en-route to endosomes, upon trans-activation by a DSL ligand expressed in signal-sending cells. Extracellular cleavage by ADAM metallo-proteases is a prerequisite for γ-secretase processing. (B) Target gene expression depends on CSL-mediated transcription derepressed by NICD. (C) After ubiquitination by a number of E3-ligases, such pool is internalized to endosomes. (D) Once in endosomes, Notch can be recycled back to the plasma membrane. (E) Alternatively, it is sorted to internal vesicles of the endosomes by ESCRTs and Lgd and eventually degraded in the lysosome. (F) A large pool of Notch is kept inactive by cisinhibition by DSL ligands. (Alfred and Vaccari, 2018[accepted]).

5.5.1.2 Mechanisms of ligand-independent Notch signalling

One of the first modifiers of Notch that appeared to act independently of ligands is the product of the Drosophila deltex (dx) gene. dx encodes a cytoplasmic RING domain-containing protein, called dx, that binds to the Ankyrin repeats of the Notch intracellular domain (Busseau et al.,
Indeed, ectopic activation of Notch signalling could be achieved by dx overexpression in *Drosophila* wing margin cells that lacked both Delta (Dll) and Serrate ligands. dx generally functions as a positive regulator of Notch signalling because dx mutants suppress Notch gain-of-function wing phenotypes (Hori et al., 2004). Molecularly, dx was found to facilitate Notch mono-ubiquitination and re-localization from the cell surface towards the late endosome (LE). It also retains Notch on the LE limiting membrane, possibly favouring ectodomain shedding in the endosomal environment. By depleting Notch from the cell surface, dx reduces the pool of receptors accessible to ligands; however, by retaining Notch on the LE surface, dx prevents some Notch receptors from degradation in lysosomes, making them available for signalling (Hori et al., 2004, 2011; Wilkin et al., 2008; Yamada et al., 2011). In addition, when complexed with kurtz (krz), a non-visual \[\beta\]-arrestin identified as the first dx physical interactor, dx attenuates Notch signalling. The binding of dx-krz complex to Notch promotes the polyubiquitination of endocytosed Notch receptors leading to their degradation. This requires the presence of shrub (Charged Multivesicular Body Protein 4 [CHMP4] in mammals), a subunit of the Endosomal Sorting Complex Required for Transport (ESCRT)-III that directs cargoes towards the intraluminal vesicles of LEs (Hori et al., 2011; Mukherjee et al., 2005). Blocking the trafficking of Notch receptors towards LEs by mutations of Rab5, Rab7, AP-3 and HOPS complex genes inhibited dx-mediated Notch activation suggesting that ligand-independent Notch signalling requires Notch receptors to be localized to the LE limiting membrane (Vaccari et al., 2008; Wilkin et al., 2008; Zheng et al., 2013). Consistent with the fact that dx mostly affects ligand-independent signalling, ectopic Notch signalling in *Drosophila* ESCRT mutant tissues is not abolished by mutations that block ligand activity (Vaccari et al., 2008). Mechanisms of ligand-independent Notch signalling are summarised in Figure 4.
Figure 4: Non-canonical Notch signalling. (A) A pool of Notch can be activated in a ligand-independent fashion (B) Such pool of internalized Notch can be saved from endosomal sorting towards degradation by the activity of the E3 ligase dx, and cleaved at the endosome, or upon fusion with the lysosome. β-arrestin, ESCRTs and other factors participate in the process. (C) Cis-inhibition prevents inappropriate ligand-independent activation. (D) In some cases this pathway can result in target gene expression that is CSL-independent. Inhibition of signaling can be achieved by direct binding to the apical determinant crb, or to Dvl, a Wnt signalling component (E). The level of a second Wnt component, β-catenin (β-cat), which also binds to Notch, is downregulated by endosomal sorting, thus preventing excess Wnt signaling. (F) Notch activation is inhibited by Akt signaling by directly binding the component PI3K, possibly in the endosomal system. (Alfred and Vaccari, 2018[accepted]).

Drosophila Suppressor of Deltex [Su(dx)] mutants dominantly suppress the phenotypes of dx mutants, hence the name. Su(dx), which encodes a Homologous to the E6-AP Carboxyl
Terminus (HECT)-type E3 ubiquitin ligase of the Neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4) family, has been described as a negative regulator of the Notch pathway by antagonizing Deltex and Notch (Busseau et al., 1994; Cornell et al., 1999; Fostier et al., 1998; Mazaleyrat et al., 2003). Su(dx) transiently interacts with Notch at the cell surface and subsequently sorts constitutively-internalized full-length Notch receptors away from the Rab11-positive recycling endosome and into an ESCRT/ubiquitin-positive compartment for degradation to downregulate signalling (Djiane et al., 2011; Wilkin et al., 2004). Su(dx) also counteracts ligand-independent Notch signalling by directly ubiquitinating Notch receptors. *Drosophila* Nedd4, a second HECT-domain E3 ligase, also reduces ligand-independent Notch signalling using similar mechanisms proposed for Su(dx) (Sakata et al., 2004). In addition, *Drosophila* Nedd4 family interacting protein (Ndfip) promotes ligand-independent Notch signalling using the same mechanism as dx (Dalton et al., 2011). The mouse homolog of Su(dx), called Itchy E3 Ubiquitin Protein Ligase (Itch), also physically interacts with Notch, promotes its ubiquitination and degradation, and subsequently downregulates Notch signalling (Qiu et al., 2000). Su(dx) may also antagonize Notch signalling by promoting the degradation of dx. Indeed, Atrophin-1 Interacting Protein 4 (AIP4), the human Su(dx) homolog, directly binds dx to promote its polyubiquitination and subsequent degradation (Chastagner et al., 2006).

Ligand-independent signalling in *Drosophila* is also prevented by *lethal (2) giant discs* (*lgd*) which was originally classified as a tumor suppressor gene because its deletion caused overproliferation of larval epithelial imaginal discs, eventually found to be due to ectopic activation of Notch signalling (Bryant and Schubiger, 1971; Watson et al., 1994)]. *lgd* encodes a C2-domain containing protein that binds phospholipids and interacts with the ESCRT-III subunit shrub. Analysis of *lgd* mutant tissues suggests that *lgd* functions in endosomal sorting towards degradation and that defects in *lgd* ultimately reduce shrub function, causing Notch
receptors to accumulate on the limiting membrane of LEs and ectopically signal, even in absence of ligands. Importantly, such activation depends on the cleavage of Notch by the γ-secretase complex and requires fusion of LEs with lysosomes (Childress et al., 2006; Gallagher and Knoblich, 2006; Jaekel and Klein, 2006; Klein, 2003; Schneider et al., 2013; Troost et al., 2012). This suggests that that Ca^{2+} release associated with fusion events, or protein degradation in the lysosomal lumen, could substitute for ligands in shedding the extracellular part of the Notch heterodimer. lgd regulation of ESCRT activity might extend beyond Notch. Indeed, Drosophila lgd mutants display ectopic activation of the BMP/Dpp signalling receptor Thickveins (Morawa et al., 2015).

Human lgd homologs Coiled-Coil And C2 Domain Containing 1A (CC2D1)A/B also play important roles in endosomal sorting by interacting with and regulating the ESCRT-III subunit CHMP4. However, CC2D1A and CC2D1B mutants do not display marked differences in Notch signalling (Drusenheimer et al., 2015; Martinelli et al., 2012; Usami et al., 2012). This, together with the evidence that human lgd paralogs also control Nuclear Factor Kappa B (NF-κB) and Epidermal Growth Factor Receptor (EGFR) signalling (Deshar et al., 2016; Zhao et al., 2010), indicates that aspects of endocytic ligand-independent Notch activation in human cells might differ from the Drosophila paradigm.

5.5.1.3 ESCRT and ligand-independent Notch signalling

Drosophila mosaic animals indicate that ESCRT function is required to regulate Notch signalling, which drives multiple cell fate decisions. Drosophila organs developing in absence of ESCRT-I, -II, -III activity display increased, and for the most part ligand-independent, Notch signalling activity, due to accumulation of Notch receptors that fail to be included into MVBs on the limiting membrane of endosomes. Signal activation does not require ligands because it still occur when ligands Delta and Serrate are impaired genetically (Herz et al., 2006; Moberg
et al., 2005a; Thompson et al., 2005; Vaccari and Bilder, 2005; Vaccari et al., 2009). Despite endosomal Notch accumulation, ESCRT-0 mutant organs of mosaic animals do not show ectopic Notch signalling activity (Jékely and Rørth, 2003; Lloyd et al., 2002; Tognon et al., 2014), highlighting differences in regulation of Notch signalling by distinct endosomal sorting components.

5.5.2 ESCRTs and other signalling pathways

Analysis of mouse ESCRT knock-outs revealed a requirement for cell survival, proliferation and signalling regulation leading to lethality early during embryogenesis (Komada and Soriano, 1999; Ruland et al., 2001; Shim et al., 2006; Yamada et al., 2002). Interestingly, a mouse hypomorph mutant of Vps25, encoding a ESCRT-II component, allows development to occur and reveals a specific requirement for ESCRTs in downregulating Sonic Hedgehog and FGF(fibroblast growth factor) signalling during limb development (Handschuh et al., 2014). Several other signalling pathways have been shown to be deregulated when ESCRT function is impaired in multiple model systems. These include JNK (Jun amino-terminal kinases), JAK/STAT (Janus kinase/signal transducer and activator of transcription), Hedgehog, Wnt, FGF, Toll, NFκβ and TGF-β (transforming growth factor) signalling (Handschuh et al., 2014; Huang et al., 2010; Jékely and Rørth, 2003; Lund and Delotto, 2011; Mamińska et al., 2016; Matusek et al., 2014; Moberg et al., 2005a; Rodahl et al., 2009; Seto and Bellen, 2006; Shim et al., 2006; Taelman et al., 2010; Woodfield et al., 2013a).

5.5.3 ESCRTs, cell polarity and apoptosis

In ESCRT mutant Drosophila tissues, cell polarization is impaired, likely because a number of polarity determinants require endosomal trafficking to be maintained at correct levels to polarize membranes and cell-cell junctions (Dukes et al., 2011; Gilbert et al., 2009; Leithe et al., 2009; Lobert and Stenmark, 2011; Palacios et al., 2005). Apoptotic response is enhanced as well. However, it is not clear whether this is an indirect consequence of the signalling and
polarity defects (Herz et al., 2006, 2009; Woodfield et al., 2013a). Ectopic activation of signalling and altered cell polarity contribute to formation of tumor-like tissue in epithelial organs of Drosophila lacking ESCRT-I, -II, -III components, that are highly over proliferative, especially when apoptosis is inhibited. These traits led to the proposal that ESCRT genes act as tumor suppressors in metazoans (reviewed in (Vaccari and Bilder, 2009)). Drosophila ESCRT-0 genes, however, do not behave as tumor suppressors, perhaps reflecting the distinct evolutionary origin of the complex or redundancy with other cargo-recruiting molecules like TOM1 or GGA proteins (Tognon et al., 2014).

Overall, development and cell biology studies in multicellular organisms reveal that ESCRTs play essential and pleiotropic functions that impact deeply tissue formation and homeostasis.

### 5.6 Roles of ESCRTs in human pathology

#### 5.6.1 ESCRTs and cancer

Misexpression of ESCRT subunits has been associated with several types of human cancers. However, the role of ESCRT in tumorigenesis remains highly controversial. One of the most studied ESCRT in this regard is the ESCRT-I gene TSG101, which was initially isolated in a search for novel tumor suppressor genes (the acronym stands for Tumor Susceptibility Gene 101). Inactivation of TSG101 in NIH3T3 cells gave rise to metastatic tumors when xenografted in nude mice (Li and Cohen, 1996). Consistent with this, TSG101 expression is significantly downregulated in cervical carcinomas (Broniarczyk et al., 2010). Despite this, the role of TSG101 as a tumor suppressor has been debated, because it was later found that conditional knock-out of Tsg101 in mouse mammary epithelia did not promote tumor formation but instead arrested cell growth (Krempler et al., 2002; Wagner et al., 2003). Although TSG101 expression seems tightly regulated by an active mechanism (Feng et al., 2000), a study evaluating the
effect of TSG101 overexpression indicated that tumor maintenance and progression, rather than initiation, might benefit from higher levels of TSG101 (Oh et al., 2007). Despite this, the gene has been found significantly overexpressed also in lung cancer (Liu et al., 2010), gallbladder adenocarcinoma (Liu et al., 2011), papillary thyroid tumors (Liu et al., 2002b) and ovarian carcinomas (Young et al., 2007a). These tumors might be addicted to high levels of TSG101, as its depletion was shown to reduce tumor growth, to slow tumor migration, to halt cell cycle progression and to trigger apoptosis of cancer cells (Young et al., 2007b; Zhang et al., 2011; Zhui et al., 2004). TSG101 appears also to be a prognostic marker in some cancers because its high expression correlates with poor prognosis, decreased survival, high tumor stage, increased metastasis and invasion (Liu et al., 2011; Young et al., 2007b).

Besides TSG101, another ESCRT-I gene, VPS37A, was identified because of its down-regulation in hepatocellular carcinomas (HCC) and named HCRP1 (human hepatocellular protein 1) accordingly (Bache et al., 2004; Xu et al., 2003). Reduced VPS37A/HCRP1 expression strongly correlates with depth of tumor invasion, lower survival and higher rate of disease recurrence not only in hepatocellular carcinoma, but also in breast cancer, renal cell carcinoma, oral and oropharyngeal cancers (Chen et al., 2015; Lai et al., 2009; Wittinger et al., 2011; Xu et al., 2014, 2003). Most of the effect of VPS37A loss has been attributed to reduced EGF receptor degradation (Bache et al., 2004; Wittinger et al., 2011), activation of downstream MAPK/ERK signalling and increased matrix metalloproteinase-2 (MMP2) expression: the loss of VPS37A has been proposed to increase tumor proliferation and invasion, and in ovarian cancer patients to lower response to cetuximab treatment (Wittinger et al., 2011; Xu et al., 2003).

Several subunits of the human ESCRT-III and Vps4 complexes have been also linked to tumor development. CHMP1A appears significantly downregulated in renal cell carcinomas (You et al., 2012) and pancreatic tumors (Li et al., 2008; Mochida et al., 2012), in which it has been
proposed to function as a tumor suppressor. Accordingly, non-tumorigenic human embryonic kidney cells acquire the ability to form xenograft tumors when CHMP1A is depleted (Mochida et al., 2012). CHMP1A overexpression inhibits the proliferation of renal (You et al., 2012) and pancreatic tumor cells (Mochida et al., 2012). CHMP1A appears to inhibit tumor growth in the pancreas by regulating the activation of ATM (ataxia telangiectasia mutated) kinase and phosphorylation of p53 (Li et al., 2008; Mochida et al., 2012). Recent reports have identified a strong upregulation (and correlation with poor prognosis) of CHMP4B in hepatocellular carcinoma, and have suggested that CHMP4B and CHMP4C might be required to sustain proliferation and resistance to anti-cancer treatment in human hepatocellular and lung cancer cell lines, respectively (Hu et al., 2015; Li et al., 2015). In a study aimed at characterizing miRNAs in exosomes of hepatocellular carcinoma cells, it was found that modulation of VPS4A changed exosome content and activity. Vps4A was also found to act as a tumor suppressor, by repressing the PI3K/Akt pathway (Wei et al., 2015). Other studies suggested that VPS4A and exosomes could influence resistance to cancer drugs like cisplatin and doxorubicin by modulating their efflux (Chen et al., 2006; Safaei et al., 2005).

Finally, expression of the ESCRT-0 component HRS is significantly increased in human tumor tissues derived from the stomach, colon, liver, cervix and melanoma — suggesting the existence of a tumor-enhancing function for HRS. Depletion of HRS reduced the tumorigenicity and metastatic ability of HeLa cells and upregulated the protein level of adherens junction component E-Cadherin (Toyoshima et al., 2007). Since HRS functions in the endolysosomal trafficking and degradation of E-Cadherin (Palacios et al., 2005; Toyoshima et al., 2007), it has been proposed that in these tumors the cargo sorting function of HRS is hijacked to downregulate E-cadherin and promote metastasis. Overall, the involvement of ESCRT in tumorigenesis is multifaceted and likely to be dependent on the tumor context, reflecting the complexity of the phenotypes observed in ESCRT mutant organs of Drosophila.
5.6.2 ESCRTs and neurodegeneration

ESCRT loss is observed frequently in many neuropathologies. Among the best characterized are the form of autosomal dominant frontotemporal dementia (FTD) caused by mutations in CHMP2B, a subunit of ESCRT-III (Skibinski et al., 2005; van der Zee et al., 2008). The mutations lead to loss of the protein C-terminus, which controls autoinhibition and interaction with Vps4 (Lee et al., 2007; Obita et al., 2007; Stuchell-Brereton et al., 2007; Urwin et al., 2010; Zamborlini et al., 2006). Accordingly, enlarged dysmorphic late endosomes have been found in cells of FTD patients (Nielsen et al., 2012; Skibinski et al., 2005). Similar endosomal phenotypes are observed when mutant CHMP2B is overexpressed in human cells (van der Zee et al., 2008). It has been proposed that mutant CHMP2B impairs endosome-to-lysosome fusion by blocking the endosomal recruitment of the GTPase Rab7, by inhibiting ESCRT-III dissociation from endosomes, or by preventing the disassembly of ESCRT-III complex. Defective autophagy is another mechanism by which CHMP2B mutations might cause FTD. Such scenario is suggested by the presence of ubiquitin inclusions positive for the autophagy marker p62, which are often observed upon failure of autophagic clearance (Cox et al., 2010; Holm et al., 2007; Lee and Dutta, 2009; Lee et al., 2007). Overall, the endo-lysosomal and autophagy defects are thought to lead to accumulation of protein aggregates, inducing neuronal degeneration, which is a hallmark of the disease. CHMP2B mutations have also been identified in amyotrophic lateral sclerosis (ALS) patients (Cox et al., 2010; Parkinson et al., 2006) suggesting that defective ESCRT activity may contribute also to ALS pathogenesis.

Mutations in the microtubule severing protein Spastin, that has been found associated to the ESCRT-III complex during cytokinesis and nuclear membrane reformation, cause hereditary spastic paraplegia (HPS) (Reid et al., 2005). Spastin function in HPS has been linked to shaping of the endoplasmic reticulum (ER) (Park et al., 2010) and recently to formation of
lipid droplets (Papadopoulos et al., 2015). This indicates that either ESCRT-independent functions of Spastin are affected in HPS or that ESCRTs and Spastin might cooperate in membrane and microtubule remodeling at the ER or in lipid droplets. Underscoring this interesting possibility, mutations in VPS37 (ESCRT-I) have also been identified in HPS patients (Zivony-Elboum et al., 2012).

While no mutations have been isolated so far, ESCRT-III function has also been reported to be important for aspects of Alzheimer’s disease (AD) and of Lewy Body Dementia (DLB, an umbrella term for two related diagnoses, Parkinson’s disease dementia and dementia with Lewy bodies). Lewy bodies are abnormal aggregates containing damaged alpha-synuclein (α-SYN) and other proteins, and α-SYN aggregation is a trait associated to the progression of Parkinson’s disease and DLB (Baba et al., 1998; Braak et al., 2003). A feature of Alzheimer’s disease and of DLB is the prion-like cell-to-cell spreading of α-SYN aggregates leading to rapid disease progression (Lee et al., 2011). According to recent studies, α-SYN aggregates are taken up by clathrin-mediated endocytosis, undergo ESCRT-mediated trafficking through MVBs, and are degraded in lysosomes (Alvarez-Erviti et al., 2011; Hasegawa et al., 2011; Spencer et al., 2016). Rapid clearance of α-SYN aggregates and amelioration of the neurodegenerative pathology was observed upon CHMP2B overexpression (Spencer et al., 2014, 2016); on the other hand, siRNA-mediated depletion of CHMP2B increased the exocytosis and intercellular transmission of α-SYN aggregates (Spencer et al., 2016). In addition, α-SYN aggregates colocalized with Vps4 (Kurashige et al., 2013), and inhibition of Vps4 function using a dominant-negative construct blocked lysosome-mediated degradation and increased extracellular secretion of α-SYN, possibly by means of exosomes (Hasegawa et al., 2011).

The formation of amyloid-beta (Aβ) aggregates in AD also appears to involve regulation by ESCRT proteins. In fact, it has been recently shown that Aβ and amyloid protein
precursor (APP) are sorted into the intraluminal vesicles of MVBs. Depletion of Hrs and Tsg101 increases the intracellular accumulation of Aβ by simultaneously inhibiting lysosomal delivery of APP and reduced Aβ secretion through a yet-unknown mechanism (Edgar et al., 2015).

Finally, early work showed that fluorescently-tagged polyglutamine aggregates of mutant Huntingtin protein required the function of ESCRT-III protein CHMP3/Vps24 for autophagic clearance (Filimonenko et al., 2007; Yamamoto et al., 2006). However, no follow-up has further detailed alterations of ESCRT activity in Huntington’s disease.

5.6.3 ESCRTs and infection

As introduced above, a number of pathogenic viruses including the human immunodeficiency virus-1 (HIV-1), the hepatitis C virus (HCV), and the Ebola virus (EBOV) hijack ESCRTs for their maturation and eventual budding to release infectious particles from infected cells. Indeed, plenty of data indicate that viral proteins, such as the Gag protein of HIV-1, recruit TSG101 and ALIX, which in turn recruit ESCRT-III and VPS4 proteins, to the neck of the viral particle assembling at the plasma membrane (Bleck et al., 2014; Corless et al., 2010; Effantin et al., 2013; Van Engelenburg et al., 2014; Garrus et al., 2001; Jun et al., 2015; Prescher et al., 2015; Sandrin and Sundquist, 2013). In the absence of TSG101 and ALIX, HCV, Herpes simplex virus type 1 (HSV-1), and to some extent HIV-1, are still able to recruit ESCRT-III (Corless et al., 2010; Morita et al., 2011; Pawliczek and Crump, 2009) suggesting that additional proteins mediate these interactions. Alternatively, viral proteins may be able to recruit downstream ESCRT components; for instance the matrix protein VP40 of EBOV, in addition to recruiting TSG101, also directly recruits VPS4 along with some other ESCRT proteins to the site of budding (Silvestri et al., 2007).
In addition, a number of reports suggest that several viruses incorporate their proteins, mRNAs or microRNAs into exovesicles of their hosts to promote their spread, to modulate immunity, or to manipulate the microenvironment (Alenquer and Amorim, 2015; Kadiu et al., 2012; Madison and Okeoma, 2015; Meckes et al., 2010; Narayanan et al., 2013; Pegtel et al., 2010; Tamai et al., 2012; Temme et al., 2010). ESCRT activity is also required for entry of rotaviruses and human papilloma virus (HPV), as these are taken up by endocytosis, sorted into ILVs and eventually released in the cytoplasm (Broniarczyk et al., 2014; Garrison et al., 2013; Li and Blissard, 2012; Pasqual et al., 2011; Shtanko et al., 2014; Silva-Ayala et al., 2013; Simon et al., 2009). Finally, a role for ESCRT-II in the replication of HIV-1 has also been reported. In fact, depletion of ESCRT-II subunits in HIV-1-infected human HeLa cells affected the cytoplasmic trafficking of HIV-1 genomic RNA and reduced the expression of the HIV Gag protein (Ghoujal et al., 2012; Meng et al., 2015). Similar results were reported for the hepatitis B virus (HBV) (Stieler and Prange, 2014). Whether the function of ESCRT-II in this particular aspect of the viral life cycle corresponds to that in transport of endogenous mRNA in Drosophila (Irion and St Johnston, 2007) is currently unclear.

Several non-viral pathogens also exploit the function of ESCRTs in infecting their hosts. A genome-wide screen in Drosophila S2 and murine macrophage cells have identified that ESCRT components restrict the growth of mycobacteria by impairing phagosome maturation, raising the possibility that mycobacteria may disrupt host ESCRT function for their growth. Indeed, a protein secreted by Mycobacterium tuberculosis (which causes tuberculosis) binds to Hrs ofESCRT-I to hinder sorting towards the lysosome for degradation (Agaisse et al., 2005; Mehra et al., 2013; Philips et al., 2008). A subunit of anthrax lethal toxin secreted by Bacillus anthracis is packaged into ILVs of multivesicular endosomes of infected cells, both for longer half-life and for exosomal secretion (Abrami et al., 2013). Finally, Candida albicans, an opportunistic fungal pathogen that colonizes mucosal surfaces, requires ESCRT activity for
pathogenesis and colonization. In contrast to viruses and other pathogens that hijack the host ESCRT machinery, *C. albicans* uses its own ESCRT complex to adapt to the neutral-alkaline pH of the host environment (Cornet et al., 2005; Kullas et al., 2004; Wolf and Davis, 2010; Wolf et al., 2010; Xu et al., 2004; Zhang et al., 2015b). In summary, pathogens clearly exploit a wide-range of the diverse cell biologic functions of ESCRTs offering multiple points of entry for future innovative therapies. Overall these studies clearly suggest that defects in endosomal sorting, autophagy, exosome release and Spastin-dependent membrane remodeling, contribute to key aspects of the pathology of a broad range of neurodegenerative diseases and that future detailed understanding and modulation of ESCRT activity could provide a major therapeutic benefit.

5.6.4 *Other diseases linked to ESCRT function*

Mutations in the ESCRT-III subunit, CHMP4B, have been identified in progressive childhood posterior subcapsular cataracts (PCPSC) linked to chromosome 20q (Shiels et al., 2007). According to Sagona and colleagues, CHMP4B may protect eye lens from developing cataract by mediating the autophagolysosomal degradation of micronuclei during lens differentiation, or by ensuring efficient cytokinesis (Sagona et al., 2014). Intestinal epithelial cells (IECs) of patients with Crohn’s Disease, an inflammatory bowel disease, possess significantly upregulated Vps4B expression. This upregulation facilitates apoptosis of IECs by activating the MAPK signalling pathway (Zhang et al., 2015a).

5.7 *Aim of the work*

The aim of this study was to identify novel biological roles of ESCRT using *Drosophila* as a model due to its genetic tractability. Here, we focused on the ESCRT-II subunit, *Vps25*, because Drosophila epithelial tissues lacking *Vps25* display tumour-like phenotypes including uncontrolled signaling, overproliferation and loss of apico-basal polarity (Herz et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005; Woodfield et al., 2013b).
Objectives

i. Define the set of genes that genetically interact with ESCRT-II subunit Vps25 and the range of biological processes that influence or are influenced by ESCRT function in epithelial tissue.

ii. Identify those Vps25 interactors that also participate in tissue growth.

iii. Characterize novel interactors, focusing on that might clarify the involvement of ESCRTs in cancer and neurodegeneration.

Parts of this chapter may have been published before completion of the whole thesis
6 MATERIALS AND METHODS

6.1 Fly strains, mapping, and genetics

Flies were maintained on standard yeast/cornmeal/agar media. All experiments were performed at 25°C with dry yeast added to the surface. Other genetic markers and special chromosomes are described by FlyBase (FlyBase Consortium 2003). Deficiency kits, P-element insertion lines and all mutant lines were obtained from the Bloomington Centre, unless otherwise stated. RNAi lines were obtained from either the Bloomington or Vienna Drosophila RNAi Centre (VDRC).

6.2 Genetic screen

The fly strain previously generated in the lab MS1096GAL4, UAS-Vps25RNAi#3/FM7 was used to set up crosses with deficiency lines. Within these deficiency regions, high-ranking mutant lines available in the stock centre were ordered and tested. RNAi or UAS-based strains were crossed to MS1096GAL4 driver alone to screen for non-specific effects on wing phenotypes. The size of the wing was used to determine the modification. Because, we observed a variability of 10% in the baseline wing phenotype, only deficiencies/mutants that modified the wing size by 20% or greater were considered. Adult flies were scored 2-3 days after eclosion and only females were considered due to the sex-based difference in size.

More than 10 females from crosses displaying modification were collected in isopropanol and kept at -20°C until preparation. Wings were dissected in isopropanol and mounted on microscopy slides using a mixture of Canadian balm (xylem-free) with methyl salicylate 1:1. Preps were dried at room temperature and analyzed and imaged with a Nikon SMZ1500 microscope using the NCIS Elements 5.0 software. For the secondary screen, we generated the eyGAL4, UAS-Delta#2/CyO stock using standard genetic recombination procedures. Dop mutant stocks and UAS constructs were obtained from Arno Muller lab.
6.3 CRISPR/Cas9 mutagenesis

For the design of guide RNAs we used the MIT CRISPR design tool (http://crispr.mit.edu/). We selected those gRNA sequences that had fewer predicted off-targets that fell on non-target chromosomes. To express Cas9 in Drosophila germ cells, we used a previously constructed pBFv-nos-Cas9 plasmid 140, containing the wild-type Cas9 coding sequence, 1264 base pairs of the nanos promoter, which has been shown to drive highly specific germline expression, and the nanos 3’UTR. The attB donor sequence for site-specific integration by the PhiC31 system, the ampicillin resistance cassette and the vermilion gene as an eye pigmentation marker are also included in the pBFvnos-Cas9 plasmid. The general sgRNA expression vector pBFv-U6.2 140, obtained from the NIG-Fly stock center, contains 399 base pairs of the Drosophila snRNA:U6:96Ab gene promoter sequence and 81 base pairs of the sgRNA scaffold. The attB donor sequence, the vermilion marker and an ampicillin resistance cassette are also incorporated into the transformation vector. The sgRNA target sequences, selected as 20 nt sequences preceding a NGG PAM sequence in the genome, were inserted between two BbsI restriction sites at the beginning of the sgRNA scaffold and overhangs were added to allow ligation. The oligonucleotides used to construct the sgRNA vectors are as follows:

- **CG11876**
  - F: 5’ CTTCGACCTGGAACCTTCTCCATGC 3’
  - R: 5’ AAACGCATGGAGAAGTTCCAGT 3’

- **CG12163**
  - F: 5’ CTTCGTAAGAATCAGGGATCCTG 3’
  - R: 5’ AAACCAGGATCTTGATCTTTCAC 3’

- **CG3764**
  - F: 5’ CTTCGCACACGCACTTTTCTGC 3’
  - R: 5’ AAACGTACGAAAAGCTGTCGC 3’

- **CG10147**
  - F: 5’ CTTCGGGCATGCGGTTTGTCTGC 3’
  - R: 3’ AAACGCCAGACAAACCGCATGCCC 5’

- **Mkrn1**
  - F: 5’ CTTCGCTTGGGATGCGCATTTCCAGCAC 3’
  - R: 5’ AAACGTGCGTACCGACTCCAGC 3’

- **CG32113**
  - F: 5’ CTTCGTGGGTGCTGAACACGTATC 3’
Plasmids containing gRNA were sent to Genetic Services Inc. to perform site-directed injections. All the constructs were injected into embryos for integration of sgRNA vector into the attP40 landing site on the second chromosome.

The T7 E1 endonuclease assay was used to screen for indel mutants. Briefly, 15 µl from the 50 µl total volume of PCR product was taken and transferred in new PCR 0,2ml eppendorf tube. The products of the PCR reaction were denatured and reannealed to facilitate the formation of heteroduplex between DNA strands. Then 10 µl from the reannealed PCR product was removed and transferred into a new eppendorf tube to start the digestion. In the same tube 2µl of 10X NEBbuffer 2 (provided by New England Biolabs), 0,25 µl of T7E1 and 7,75 µl of nuclease free water were added reaching the total volume of 20µl. The sample was incubated for 20 minutes at 37°C. Then immediately 15 µl of the total volume was transferred into a new tube and 3 µl of 6X loading dye was added, the fragment analysis was concluded by loading the 18 µl volume on a 1.5% 1XTAE agarose gel.

6.4 Lifespan and climbing assays

For the lifespan experiments, 120 flies (unless otherwise stated) collected at day 1 of their lives were housed in groups of 20 and the flies were transferred every 2 days onto fresh food and the number of dead flies recorded. For oxidative stress treatments, hydrogen peroxide was mixed into the fly medium at a final concentration of 3%. Groups of ten flies were placed in an empty climbing vial and then tapped down to the bottom. They were allowed for 15 seconds to climb past a line marked 5 cm from the bottom of the vial. The number of flies above the 5-cm mark within 15 seconds was recorded as a percentage of flies able to climb the tube. The day before the assay, flies were transferred to a new food vial to help reduce wet food from inhibiting their climbing ability; moreover, flies were not exposed to CO2 at
least 24 hours before the assay, as carbon dioxide quickly anesthetizes the insects. The climbing assay was performed on minimum of 30 flies per genotype at different ages.

6.5 DQ BSA red assay

DQ Red BSA is a fluorogenic substrate for proteases and is used as a readout of lysosomal function. Upon hydrolysis DQ Red BSA releases fluorescent fragments that have excitation and maxima emission of ~590 nm and ~620 nm. Third instar larvae were dissected to expose the internal organs and placed in an eppendorf tube containing 20µg/µl of DQ Red BSA diluted in M3 medium. The samples were incubated for different time (min 0 hour to 5 hours) at RT, then were fixed with 4% PFA, washed 3 times for 5 minutes with PBS 1X and then only wing discs were mounted on a glass slide with Mowiol. For the negative control, bafilomycin A1 500nM was added in samples together with the DQ Red Bsa, to inhibit lysosomal degradation. The samples were then analysed under a confocal microscope.

6.6 RNA extraction and qPCR analysis

RNA was obtained from fly tissues using the RNeasy kit (QIAGEN). cDNA was synthesized using SuoerScript VILO (Life Science Technologies) and qPCR primers were designed using the Universal Probe Library (UPL) Roche.

6.7 Cell culture

MCF10A cells were cultured in DMEM/F12 (1:1) supplemented with 5% Horse Serum (Invitrogen), 10 µg/ml Insulin, 0.5 µg/ml Hydrocortisone, 100 ng/ml cholera toxin (SIGMA) and freshly added 20 ng/ml EGF. All cells were cultured at 37 °C and 5% CO2 in a humidified incubator. To knock down genes, siRNAs against desired genes were transfected into MCF10A cells using the lipofectamine RNAiMax transfection reagent (Life technologies) by following the manufacturer's instructions.
6.8 **Protein extraction and Western blotting**

The samples were homogenised on ice in a 1.5 µl sterile eppendorf tube containing the LBPI buffer (RIPA lysis Buffer + proteinase inhibitor 1:100) with autoclaved dispensable pestles. Depending on the tissues, 60µl of LBPI for each fly or 35µl for 10-15 heads was added. The tubes were left on ice for 30 minutes, and the homogenate was centrifuged at 13200 rpm for 20 minutes at 4°C. The supernatant was finally transferred into a new tube and stored at -20°C. Western blotting was performed according to standard techniques. Antibodies used are: Rabbit anti-Ref2p (gifted by T Rusten) 1:1000, rabbit anti-p62, Rabbit anti-Atg8 (gifted by Gabor Juchaz) 1:1000, Mouse anti-Tubulin 1:10000.

6.9 **Immunostainings**

Imaginal discs were fixed with 4% paraformaldehyde (PFA) diluted in H2O for 20 minutes. Then, samples were rinsed three times with 0.1% triton PBS1x for 5 minutes. For permeabilization, samples were treated for 10 minutes with 1% triton PBS1x and then the blocking solution, composed of 5% BSA in 0.1% triton PBS1x, was added for 30 minutes. Samples were incubated with primary antibodies diluted in blocking solution overnight (O.N.) at 4°C. Three washes in 0.1% triton PBS1x of 5 minutes each were performed. Secondary antibodies, diluted in PBS1x, were added for two hours at room temperature (RT). Samples were then rinsed three times for 5 minutes with 0.1% triton PBS1x. Eventually, DAPI was added for 10 minutes and then washed once with PBS1x. Samples were then mounted with Glycerol or Moviol (Calbiochem). The antibody used was mouse anti-NICD (DSHB C17.9C6) at dilution 1:100. Images were obtained with TCS microscope (Leica, Heidelberg, Germany) using 20x/NA 0.5, 40x/NA 1.25 or 63x/NA 1.4 oil immersion lenses.
6.10 Bioinformatic and data analysis

Protein interactome data were obtained from the GeneMania database which integrates protein-protein interaction data from other sources (Mostafavi et al., 2008). Interactions are visualized in Cytoscape (Shannon et al., 2003) and Gene Ontology enrichment analysis were performed using the DAVID suite. Homology alignments were made using the MEGA suite (Kumar et al., 2016) after obtaining FASTA sequences from the NCBI database. Graphs were made and data were statistically analysed using either R statistical software or GraphPad Prism.
7 RESULTS

7.1 Screening for Vps25 modifiers

7.1.1 Knockdown of Vps25 induces a small deformed wing phenotype in adult flies

To identify genes that modulate ESCRT activity in the wing disc, we utilised a hypomorphic knockdown of Vp25 by RNAi under the MS1096GAL4 driver, which directs expression in the dorsal wing disc compartment. A line expressing this dsRNA was generated by recombining the driver and a UAS Vps25 RNAi both mapping to the X-chromosome. Differently from generation of Vps25 null tissue in imaginal discs (Vaccari and Bilder, 2005), expression of the Vps25 RNAi line under MS1096GAL4, does not result in early pupal lethality indicating that depletion of Vps25 results in a hypomorphic phenotype. In contrast, reduction of Vps25 expression leads to formation of small wings that are slightly bent upwards. Such phenotype is specific because: (1) two independently-generated MS1096, Vps25RNAi lines produced similar phenotypes; (2) further reduction of Vps25 gene dosage by heterozygosity for the null Vps25A3 allele in the MS1096, Vps25RNAi background enhanced the phenotype leading to a further reduction in wing size; (3) expression of a UAS-Vps25 in the MS1096, Vps25RNAi background suppressed completely the phenotype leading to formation of wild-type wings; (4) reducing the gene dosage of AGO-2, a positive regulator of RNA interference suppressed the small wing phenotype (Figure 5A). The identification of a Vps25 hypomorphic phenotype, which is so far unreported, allowed us to establish a sensitive background to screen for modifiers that genetically interact with Vps25 (Figure 5A, B).
Figure 5: Vps25 knockdown causes a small wing phenotype sensitive to gene dosage. (A) The small wing phenotype is caused by specific reduction of Vps25 levels via RNAi. (B) Scheme showing the crossing strategy used for the screening. (C) Mutant alleles of trafficking genes modify the Vps25RNAi phenotype. (D) Vps25RNAi phenotype is sensitive to gene dosage of signalling pathway components.

7.1.2 Phenotypic modification by trafficking and signalling genes

Generation of Vps25 null mutant tissue in Drosophila imaginal discs has been shown to cause defects in tissue proliferation and polarity, primarily because of defective endosomal sorting and aberrant signalling, the latter being a consequence of the former. We therefore reasoned that halving the gene dosage of genes that encode components of endosomal sorting and signalling pathways should modify the Vps25RNAi phenotype, if these processes also contribute to our phenotype. To test whether the Vps25RNAi phenotype is caused by defects in endosomal sorting and trafficking, we crossed Vps25RNAi to mutants for ESCRT (Hrs, STAM, ept or tsg101, vps32, vps2, vps28) or other trafficking genes (the clathrin genes Chc and Clc, the syntaxins Syx7 and Syx13, the Rab family genes Rab5, Rab8, Rab28). In both cases, we observed modification of the Vps25RNAi phenotype (Figure 5C). Studies have
shown that the defects associated with ESCRT mutations is partly due to aberrant activation of signalling from receptors localised in the endosomes, and that fail to be degraded in the lysosome (Moberg et al., 2005b; Thompson et al., 2005; Vaccari and Bilder, 2005). This might explain why mutants for genes like Rab5, avl that regulate earlier endocytic steps, specifically the formation of early endosomes, suppress the Vps25RNAi phenotype, possibly by preventing the arrival of receptors in compartments where they can be ectopically activated. The situation is, however, not clear with ESCRT mutants. Indeed, we found that some ESCRT genes act as suppressors and others as enhancers, possibly underlining the complexity and diversity of ESCRT activity.

In vps25 mutants Notch is ectopically activated in a ligand-independent and γ-secretase- dependent fashion (Herz et al., 2006; Vaccari and Bilder, 2005). This suggests that part of the phenotype of Vps25RNAi might be due to increased Notch signalling activity and that reduction of gene dosage of Notch signalling components might modify the wing phenotype. As expected, crossing to a Notch null allele suppressed the phenotype strongly. In fact, null alleles of positively-acting Notch signalling pathway all suppress the phenotype, confirming the ectopic Notch activation observed in the Vps25 null mutants.

Upd and Dome, the JAK kinase hopscotch (hop) and the transcriptional activator STAT92E are essential components of the JAK/STAT signalling pathway in Drosophila. Ligand-binding causes receptor dimerization, activating the associated JAK. JAK-mediated phosphorylation of the cytoplasmic receptor-tail provides binding of cytoplasmic Stat92E. Phosphorylation of bound STAT by JAK results in release and translocation of a STAT-homodimer into the nucleus where it functions to transcribe target genes (Luo and Dearolf, 2001). Vps25 null tissues in the eye disc display overactivation of JAK/STAT signalling due to Notch-induced overexpression of Upd (Moberg et al., 2005b; Vaccari and Bilder, 2005). Crossing the Vps25RNAi line with mutant alleles for JAK/STAT pathway components leads
to suppression of the small wing phenotype; this suggests that similarly to $V_{ps}25$ mutants, reduction of $V_{ps}25$ leads to overactivation of JAK/STAT signalling. The TGF-$\beta$/Dpp pathway is ectopically activated in imaginal discs that lack ESCRT activity (Thompson et al., 2005). The fact that reducing the gene dosage of $Sara$ and $thickveins$ ($tkv$), both positive components of the pathway, leads to suppression of the small wing phenotype suggests that overactivation of the TGF-$\beta$ signalling pathway contributes towards the $V_{ps}25RNAi$ phenotype. On the other hand, we unexpectedly observed that components of the RAS/RAF/MAPK and Wnt/Wg pathways enhance the $V_{ps}25RNAi$ phenotype (Figure 5D). This contrasts with results showing Ras and Wg activation in $V_{ps}25$ mutant tissues (Thompson et al., 2005; Vaccari and Bilder, 2005)

Modification by trafficking components suggest that the use of the $V_{ps}25$ sensitized backgrounds will allow to isolate known and new trafficking genes connected with ESCRTs. Moreover, suppression by Notch pathway components indicates that as in the null $V_{ps}25$ null mutant tissue, Notch is ectopically activated $V_{ps}25RNAi$ wings. Finally, the fact that the $V_{ps}25RNAi$ wings are reduced in size, despite activation of proliferative Notch and JAK/STAT signalling, strongly suggest that, as in $V_{ps}25$ null mutant tissue, apoptosis is active in $V_{ps}25RNAi$ wings.
Figure 6: Breakdown of screening strategy. (A) We used a deficiency screening approach to isolate modifiers, starting with larger deficiencies, to smaller deficiencies to individual loci. The image shows a section of chromosome 3R. (B) Workflow of the screening approach. (C) Breakdown of the positive hits identified at each pass of the screening.

7.1.3 F1 dominant modifier screen to isolate interacting loci

To identify novel interactors of Vps25RNAi, we first conducted a systematic screen to isolate modifying regions on the 2nd and 3rd chromosomes. Following the strategy already described, we crossed Vps25RNAi with an in house-customized Bloomington deficiency kit, which is a collection of molecularly mapped large deficiencies arranged to maximize coverage with smallest possible number of stocks. When modifications were observed we used a set of smaller deficiencies covering the larger ones (Figure 6A-C). This proved useful to quickly identify chromosomal regions containing modifier loci.

The deficiency kit that we have used is composed of 219 lines for chromosome 2 and 260 lines for chromosome 3; in each case, we estimated to remove > 85% of the chromosome. Altogether,
we identified 154 smaller regions that modify the Vps25RNAi phenotype. We identified loci that map to these regions and tested for modification using P-element insertions, RNAi lines and mutants for genes mapping to these loci. UAS-based lines (RNAi and EP insertion lines) were crossed to MS1096GAL4 alone as control to determine whether the wing size modification occurred independently of Vps25 knockdown, and genes that did were excluded. We have compiled a list of 204 genes that genetically modify the Vps25RNAi phenotype (Table S1).

7.1.4  Computational network demonstrates wide range of functional categories

We screened a large collection of genes, however, our screen was not saturating because: (1) non-modifying deficiencies could contain enhancers and suppressors erasing each other effects; (2) our screen was selective for haploinsufficiency effects; (3) we excluded genes that did not show robust modifications of the Vps25RNAi wing phenotype based on our 10% noise criteria and; (4) modifiers that could not be validated with at least another independent allele were excluded from the final list. However, based on available data in published literature, FlyBase and DAVID, we classified all validated modifiers into categories based on their known or predicted functions. As a confirmation that the screen strategy was in fact valid, membrane trafficking genes made up by number the largest category of previously characterised genes having members that included ESCRTs, syntaxins, Rabs, and intracellular trafficking proteins. Other classes identified include genes that encode signalling pathway components, transcription factors, chromatin modifiers, transporters and ion channels, cytoskeleton and motor proteins, metabolism regulators, and ubiquitin ligases. Interestingly, we obtained a group of 29 genes that have not yet been annotated and whose functions have yet to be described. Using protein-protein interaction data, and the GeneMania and Cytoscape tools (Mostafavi et al., 2008; Shannon et al., 2003), we created a network diagram showing a simplified interaction map. The genes have been grouped and color-coded according to their functions (Figure 7A). Additionally, we performed Gene Ontology enrichment analysis to reveal the biological processes enriched within our gene
list, and we expectedly found Notch signalling and MVB sorting processes to be enriched. We also found other processes to be enriched significantly, possibly illustrating the biological relevance of ESCRTs (Figure 7B).
Figure 7: Analysis of Vps25 interactors. (A) Network showing the categories of genes identified in the primary screen. (B) Gene Ontology analysis showing biological processes that are enriched.
7.2 Secondary screening of Vps25 modifiers for growth modulators

Previous work has demonstrated that Vps25 mutant tissues display overproliferation due to ectopic activation of Notch and other signalling pathways (Herz et al., 2006; Thompson et al., 2005; Vaccari and Bilder, 2005). We therefore designed a secondary screen to identify Vps25 modifiers that also influence tissue proliferation based on modification of a Notch-mediated overproliferation phenotype. To this end, we overexpressed the Notch ligand Delta under the control of the eyeless-GAL4 driver, which leads to eye tissue overproliferation. This background has already been used to screen for modifiers of growth and tumorigenesis in imaginal discs (Ferres-Maro et al., 2006). The eye tissue proliferation is indeed due to activation of Notch signalling because: reducing the gene dosage of positive regulators in the eyGAL4, Delta background suppressed the overproliferation phenotype while reducing the gene dosage of negative regulators enhanced the phenotype (Figure 8A-G).

Figure 8: Delta overexpression in the Drosophila eye causes overgrowth by activating Notch signalling. (A) wild type (B) eyGAL4-Delta, eye overgrowth induced by Delta overexpression. Mutant alleles of Delta (C), presenilin (D) and eyegone (E) in the eyGAL4-Delta background suppress the eye overgrowth while a mutant allele for Nedd4 enhance the overgrowth (F).

For the UAS-based alleles, we crossed them to eyGAL4 driver alone to eliminate those that gave an eye phenotype by themselves. Also, we eliminated candidates that have already been reported to modulate Notch signalling activity. We crossed the remaining modifiers to eyGAL4-Delta and
scored the F1 progeny for either enhancement or suppression of the eye phenotype. Altogether, we identified 43 novel candidates that induced robust modifications of the eyGAL4-Delta phenotype (Table 2).

Table 2: Plot showing only the Vps25 interactors that also modify Delta-mediated eye overgrowth (not to scale)

<table>
<thead>
<tr>
<th>Vps25RNAi Suppressors</th>
<th>eyGAL-Delta Suppressors</th>
<th>eyGAL-Delta Enhancers</th>
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<tbody>
<tr>
<td>• CG17364</td>
<td>• CG32109</td>
<td>• CG6498/dop</td>
</tr>
<tr>
<td>• CG17360</td>
<td>• cnc</td>
<td>• l(3)76BDr</td>
</tr>
<tr>
<td>• Iaf</td>
<td>• Hsc70-1</td>
<td>• Pep</td>
</tr>
<tr>
<td>• hh</td>
<td>• Nedd4</td>
<td>• hyx</td>
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<tr>
<td>• eyg</td>
<td>• CG10147</td>
<td>• CG11253</td>
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<tr>
<td>• pum</td>
<td>• Trc</td>
<td>• sec63</td>
</tr>
<tr>
<td>• Taf4</td>
<td>• Byn</td>
<td>• CG32109</td>
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<tr>
<td>• Aos1</td>
<td>• CG31100</td>
<td>• Pinta</td>
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<tr>
<td>• sec63</td>
<td>• bnl</td>
<td>• RhoGAP71E</td>
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<tr>
<td>• CG11253</td>
<td>• ImpL2</td>
<td>• CG7369</td>
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<tr>
<td>• Pinta</td>
<td>• toe</td>
<td>• Sup12-46</td>
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<tr>
<td>• RhoGAP71E</td>
<td>• slif</td>
<td>• Rcd5</td>
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<td>• CG12163</td>
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<td>• slif</td>
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<td>• ran-like</td>
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7.3 Genetic analysis of CG6498 (dop) in eye proliferation

One of the genes that we identified as a genetic interactor of Vps25 and modifier of eyGAL-Delta overproliferation phenotype is CG6498/dropout (dop), which at the time was not yet characterised. In fact, dop was one of the strongest enhancers of the eyGAL4-Delta eye overgrowth, a modification we validated using two independent lines, dop\textsuperscript{JF02778} and dop\textsuperscript{GL00220}, referred to hereafter as dopRNAi-1 and dopRNAi-2 respectively. Because the dop lines were UAS-RNAi lines, we checked if dop knockdown by itself altered eye phenotype by crossing the
dopRNAi-1 and dopRNAi-2 with eyGAL4 and found that dop knockdown had no effect on eye phenotype. By qPCR analysis, dopRNAi-1 and dopRNAi-2 reduced dop mRNA expression to 29±0.04% and 75±0.05% respectively. Importantly, the strength of the knockdown (or magnitude of dop mRNA reduction) correlates with the severity of eyGAL4-Delta phenotype modification (Figure 8). Taken together, the enhancement of the eye overgrowth is due to reduction of dop levels in the eyGAL4-Delta background.

**Figure 8:** Reduction of dop enhances Delta-mediated eye overgrowth by upregulating Delta expression. Knockdown of dop in the *Drosophila* eye using two RNAi lines (B, C) has no effect compared to wild type eye (A). However, dop knockdown in the eyGAL4, Delta background enhances the eye overgrowth (E, F) compared to eyGAL4, Delta alone (D).

There are two possibilities as to how reduced dop levels influence growth in the eyGAL4-Delta background: (1) direct effect on the Notch pathway by potentiating Notch signalling or (2) indirect effect on growth by acting on downstream effects of ectopic Notch signalling. Notch activation leads to activation of the E(spl) family of transcription factors, and this is true also of the *Drosophila* eye disc. Hence, we tested if reduced dop levels altered the expression of the *E(spl)mβ* and in fact, there is a doubling in the transcription of *E(spl)mβ*. Importantly, the
magnitude of \(E(spl)m\beta\) negatively correlates with the levels of \(dop\) mRNA \((r^2 = -0.989,\) using the R cor function). This increase in \(E(spl)m\beta\) suggests the effect on growth might partly be due to a direct effect on activation of the Notch pathway. A direct effect on Notch pathway can be caused by (1) increase in \textit{Notch} expression; (2) increase in the amount of ligand; (3) increase in the cleavage of the Notch receptor; or (4) non-canonical activation of \(E(spl)m\beta\) independently of Notch signalling. We have checked for changes in the transcript levels of \textit{Notch} and \textit{Delta}: reduced \(dop\) levels in the \textit{eyGAL4-Delta} background does not alter \textit{Notch} expression but significantly increased the expression of its ligand, \textit{Delta} (Figure 8A-G). We already know that the \textit{eyGAL4-Delta} system is sensitive to levels of \textit{Delta}; therefore, the upregulation of \textit{Delta} might be responsible for the enhanced growth of \textit{eyGAL4-Delta} eye tissue by \(dop\) downregulation.

\textit{Drosophila} dop protein is composed of three conserved domains: AGC kinase, PDZ (PSD95, Dlg, ZO-1) and DUF1908 (domain of unknown function 1908). To test which of the dop domains is important for its role in growth regulation, mutants of \(dop\) (Figure 9). These lines have already been generated by (Hain et al., 2014). Most dop mutants reproduced the effect of the \(dop\) RNAi lines, with \(dop'^1\), a missense mutant in the kinase domain, giving the mildest effect (Figure 10A-F). Overall, these experiments indicated that \(dop\) can control eye disc growth by acting on \(Dl\) levels.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{dop_domains.png}
\caption{Location of \textit{dop} mutations (Hain et al., 2014)}
\end{figure}
Figure 10: Mutant dop alleles enhance Delta-mediated eye overgrowth. Mutant dop alleles enhance Delta-mediated eye overgrowth. (A) eyGAL4, Delta (B) eyGAL4, Delta/+; dop¹/+ (C) eyGAL4, Delta/+; dop⁷/+ (D) eyGAL4, Delta/+; dop⁹/+ (E) eyGAL4, Delta/+; dop¹⁰/+.

7.4 Dop is the Drosophila homologue of mammalian MAST kinases

To identify the mammalian homologues of dop, we used the NCBI BLASTp tool, which revealed that it is the homologue of the mammalian microtubule-associated serine/threonine-kinases (MAST) MAST-1, -2, -3 and -4. FASTA files of multiple alignments of downloaded protein sequences were created with the MUSCLE software which is part of the MEGA7.0 suite (Edgar, 2004a, 2004b; Kumar et al., 2016) to identify the conserved domains. By specifically aligning the three dop domains with those of human MAST kinase paralogues, it is evident that the between Drosophila and humans, the kinase (KIN) domain is the most conserved while the DUF1908 (DUF) domain is the least conserved domain (Figure 11).
Figure 11: Homology alignment of dop to mammalian MAST kinases 1-4 across the three domains

7.5 Analysis of mammalian MAST2 in human epithelial MCF10A cells

To characterise the mammalian MAST kinase family, expression levels of the individual MAST kinase members were assessed by qPCR across various mammalian cell lines and MAST2 was found to be the most expressed while MAST1 and MAST4 are the least expressed (Figure 12A). MCF10A, a cell line derived from the breast epithelium was selected for further analysis because they endogenously express Notch receptors and ligands.
To understand if the role of MAST kinase genes in Notch signalling conserved in mammals, MAST2 expression was downregulated in MCF10A cells using siRNA and the effect on downstream Notch signalling was assessed by Hes1 expression levels. Transfection of 10nM siRNA against MAST2 reduced MAST2 expression by 60 percent, however, we did not observe any change in the expression of Notch downstream targets (HES1, HEY1), or ligands (DLL1, JAG1) or Notch receptors themselves (NOTCH1, NOTCH2, NOTCH3) (Figure 12B). There are 4 MAST paralogues in mammals and the absence of an effect on the Notch pathway may be due to redundancy among the MAST paralogues. This is unlikely because of the following reasons: (1) In MCF10A, MAST2 is the most expressed of the MAST family members; (2) In other studies where MAST2 function was reduced either by RNAi or by using dominant negative constructs, no redundancy was observed (Ren et al., 2013; Xiong et al., 2004). Together, these results suggest that MAST2 likely has no effect on Notch signalling in human epithelial cells. However, we cannot rule out the role of the other MAST kinases in Notch signalling, or differences that exist between cell types/lines.
Figure 12: Analysis of MAST expression across cell lines, and effect of knockdown in MCF10A cells (A) Expression profiling of MAST2 expression across human cell lines reveal that MAST2 is the most expressed family of the MAST kinases. (B) MAST2 knockdown in MCF10A does not alter the expression of Notch ligands, receptors, or targets. (C) MAST2 knockdown in MCF10A cells causes significant upregulation of MMP1, MMP9 and mild increase in FN1 expression.

The enhancement of *Drosophila* eye overproliferation by dop knockdown suggests that it may play an anti-proliferative role. However, a study of human cancer cell lines carrying MAST1 and MAST2 mutations suggests that they play a growth-promoting or pro-tumorigenic role (Clay et al., 2013; Robinson et al., 2011). To test whether MAST2 might play a tumorigenic role independent of Notch signalling, we selected a panel of genes that have been shown to be transcriptionally altered during tumorigenesis and we analysed their expression levels upon MAST2 knockdown in MCF10A cells. These genes were mostly chosen from an in-house qPCR reaction panel and they include: *CDH1* (E-cadherin), whose expression is frequently downregulated in breast cancer and other cancers, and is one of the main events responsible for dysfunctional cell-cell adhesion, invasiveness, and metastasis [reviewed in (Berx and Van Roy, 2001)]; *EGFR* (epidermal growth factor receptor), whose increased expression positively correlates with tumour growth and aggressiveness in some cancers (Rimawi et al., 2010); *FN1* (fibronectin 1), which is upregulated transcriptionally during metastasis of colorectal, renal, ovarian (Chaves et al., 2012; Sudo et al., 2013; Waalkes et al., 2010); *MMP* (matrix metalloproteases)-1, -2, -7, -9, which are often overexpressed in cancers and often correlates with disease progression, invasiveness and metastasis (Basu et al., 2015; Cheng et al., 2008; Decock et al., 2007; Kohrmann et al., 2009; Kousidou et al., 2004; Luo et al., 2005; Wu et al., 2014) (5) MYC, frequently mutated and found to be overexpressed in many cancers (Bièche et al., 1999; Sato et al., 1994); *CCND2*, usually hyper methylated and expressed to a lesser extent in tumours; Other genes we tested include *PTCH, CCND2, CDC25A, CDKN1A, VIM* and *P53* (Evron et al.,
2001; Fischer et al., 2002; Rodriguez-Magadán et al., 2008; Sudo et al., 2013). No changes in the expression of these genes was observed by qPCR except MMP1 and MMP9. There also seemed to be an increase in FN1 expression, although this change did not reach the statistical significance threshold (Figure 12C). These data suggest that in contrast to Drosophila, human MAST2 might modulate aspects of tumorigenesis related to cell invasion.

7.6 Large scale CRISPR/Cas9 mutagenesis of uncharacterised modifiers

7.6.1 Selection of the modifiers to mutate

29 of the modifying genes identified in the Vps25 primary screen have neither been previously studied or associated with any molecular process or function, and 14 of these also modified the eyGAL4-Dl eye overgrowth phenotype. Therefore, we selected six genes that scored positive in the primary and secondary screen for CRISPR/Cas9-directed mutagenesis based on a mix of the following criteria: (1) strength of the modification, determined quantitatively and/or visually; (2) presence of mammalian homologues, obtained by BLAST sequence alignment; (3) association of the human homologues with any disease phenotype, based on information from the DISGENET and OMIM databases and; (4) absence of mutant lines in the FlyBase database.

The six selected genes are briefly described below: (1) CG10147, whose mutation suppressed Vps25RNAi phenotype and strongly enhanced the eyGAL4-Dl eye overgrowth. In fact, CG10147 displayed one of the strongest eye overgrowths in the eyG4-Dl background. CG10147, based on domain homology, encodes a zinc finger containing protein. In this case though, CG10147 does not have a clear human orthologue; however, it was selected because of the very strong growth modification and because we validated the modification using 3 independent RNAi lines; (2) CG11876 — a suppressor of both Vps25RNAi and eyGAL4-Dl phenotypes — encodes a protein that appears to be a central hub connecting several trafficking proteins using available protein-protein interaction data. Homology alignment with the mammalian orthologues suggests that CG11876 codes for pyruvate dehydrogenase, a mitochondrial enzyme, whose mutations
cause an autosomal recessive disease called pyruvate dehydrogenase E1-beta deficiency (Okajima et al., 2008). Whether defects in endosomal trafficking contributes towards this pathology is presently unclear. (3) *CG12163*, encodes a protein that shares strong homology with mammalian *Cathepsin F (CTSF)*. Cathepsins mostly function in the lysosomes to hydrolyse and digest lysosomal contents. *CTSF* mutations have been shown to cause a type of neuropathology referred to as adult-onset neuronal ceroid lipofuscinosis (or Type B Kufs disease) (Smith et al., 2013; Tang et al., 2006a). Also, it is a suppressor of both *Vps25RNAi* and *eyGAL4-DI* phenotypes. (4) *CG3764*, encodes the *Drosophila* homologue of mammalian folliculin-interacting proteins 1 and 2 (FNIP1/2); FNIP1 and FNIP2 are two of the seven proteins that have been demonstrated to interact with folliculin (FLCN), and they are both essential for folliculin function (Hasumi et al., 2015; Takagi et al., 2008). Mutations in folliculin have been shown to be responsible for the Birt-Hogg Dube syndrome, a rare disease characterised by small benign skin lesions (folliculomas), lung cysts and kidney cancer (Chen et al., 2008; Hudon et al., 2010). FLCN, by being a part of the Tor/TSc pathway plays important roles in cell/tissue growth, autophagy, and organismal lifespan (Gaur et al., 2013; Hasumi et al., 2008; Piao et al., 2009). (5) *Mkrn1*, orthologue of mammalian *Markorin E3* ubiquitin ligase, have not been characterised sufficiently in *Drosophila* but have been demonstrated to regulate telomerase activity, p53 stability and gene transcription in mammalian systems (Kim et al., 2005; Lee et al., 2009a; Omwancha et al., 2006; Salvatico et al., 2010). (6) *CG32113*, homologue of mammalian Vps13D belongs to the Vps13 family of genes that in yeast have been described to function in Golgi to pre-vacuole trafficking (Bryant and Stevens, 1998; Stack and Emr, 1993).

### 7.6.2 Generation of knock-out mutants

For the design of guide RNAs we used the MIT CRISPR design tool ([http://crispr.mit.edu/](http://crispr.mit.edu/), Zhang Lab, MIT). We targeted gene regions that code for predicted functionally important domains, because it has been demonstrated that this approach yields stronger phenotypes
compared to the targeting of regions outside these domains (Shi et al., 2015). For CG10147, we selected exon 2, which codes for the zinc finger domain; for CG12163, exon 4 which codes for the first part of the peptidase domain; for CG3764, exon 5, expected to encode the region predicted to bind folliculin based on mammalian structural analysis; for CG11876, exon 2, predicted to code for a part of the transketolase domain.

For CG10147, we selected exon 2, which codes for the zinc finger domain; for CG12163, exon 4 which codes for the first part of the peptidase domain; for CG3764, exon 5, expected to encode the region predicted to bind folliculin based on mammalian structural analysis; for CG11876, exon 2, predicted to code for a part of the transketolase domain.

Briefly, we cloned the gRNA into pBFv-U6.2 vectors, integrated them into the fly genome at chromosome 2. Transgenic male flies carrying the gRNA and female flies carrying the nos-Cas9 (nos is a germline specific driver) were crossed to together and the resulting progeny was analysed for presence or absence of mutations (Kondo and Ueda, 2013) [Details are described in the Materials and Methods section]. We isolate the intended mutations, we used 3 approaches: (1) T7 E1 endonuclease assay to identify large insertions or deletions (indels) that cause heteroduplexes after annealing DNA strands; (2) DNA sequencing to ascertain the mutation type and status and; (3) Genetic complementation tests, useful only in cases where the mutation causes lethality when homozygous.

We started out initially using the T7 endonuclease assay to screen potential mutants and identified potential indels in 22 of 43 stocks for CG12163, 5 of 44 stocks for CG10147, 23 of 28 stocks for Mkrn1, 19 of 40 stocks for CG3764 (Figure 13). DNA sequencing on amplified regions flanking the Cas9 cleavage site was performed on selected mutants for confirmation. Since the flies at this stage were heterozygous, having mutant and wild type chromosomes, we observed the presence of double peaks beginning at the Cas9 cleavage position. We also sequenced a sample of other stocks that did not score positive for the T7 E1 assay, that is, those with single bands, and found that some of them were indeed mutants having single base deletion/insertion, resulting in a frameshift, and predicted to yield a truncated protein. This indicates that, in our hands, the T7 E1 assay might not be sensitive enough to detect small
indels. Thus, for further screening of mutants, we ignored the T7 E1 assay and focused on DNA sequencing.

Except for *Mknl* and *CG3764*, homozygous mutants of the other genes were viable. For the homozygous mutants, DNA sequencing was reperformed and the region surrounding the Cas9 cleavage site was aligned with the corresponding wild type region to validate the mutation. Mutant alleles for *Mknl* and *CG3764* failed the complementation test when crossed to their corresponding deficiency lines, confirming that their mutations were indeed homozygous lethal. Mutant lines for all genes were established and stocked.

![Figure 13: T7 endonuclease assay to identify indel mutants generated by CRISPR/Cas9. Presence of triple bands per lane suggests the presence of indels greater than 3 bases.](image)

**7.7 Preliminary characterisation of *CG12163* mutants**

**7.7.1 *CG12163* mutations cause reduction in *CG12163* gene expression and potentially lead to loss of the peptidase domain**

We focused on *CG12163* for further characterisation because: (1) being a cathepsin F orthologue, it most likely functions in the lysosome and may directly or indirectly influence
downstream endocytic trafficking outcomes including signalling (Sorkin and von Zastrow, 2009; Turk et al., 2012); (2) the human orthologue cathepsin F causes an adult-onset form of neuronal ceroid lipofuscinosis (NCL), called Type B Kufs disease, whose mechanisms are presently unclear (Di Fabio et al., 2014, 2015; Smith et al., 2013). We observed that CG12163 is expressed predominantly in the fly head compared to the rest of the other tissues, in agreement with available expression data from FlyBase, also suggesting that its role in neuronal health may be conserved in Drosophila.

**Figure 14: Description of CG12163 mutants.** (A) CG12163 is predominantly expressed in the adult fly head compared to the rest of the body. (B) Most of the mutations cause reduction in CG12163 transcript levels possibly due to mRNA decay. (C) CG1216317, CG1216318, CG1216325 mutations are predicted to encode a truncated protein lacking the functional peptidase domain.

All the CTSF mutations associated with this form of NCL occur in the peptidase domain except one and in vitro biochemical analyses have shown that the enzyme resulting from all these mutants lead to reduced peptidase activity (Smith et al., 2013) (Peters et al., 2015). Because these evidences suggest that the disease results from loss of peptidase activity, our CG12163 CRISPR/Cas9 mutants targeting the peptidase domain might represent models of the disease in flies. Based on DNA sequencing of the mutants, we selected those predicted
to lack the peptidase domain. We test if these mutations affect mRNA transcript levels by qPCR analysis because the exon position of premature termination codons have been shown to affect mRNA decay (You et al., 2007). CG12163\textsuperscript{17}, CG12163\textsuperscript{18} and CG12163\textsuperscript{25} were selected for further analysis because they reduce the mRNA levels of CG12163 by 89%, 81% and 85% respectively. Also, the residual mRNA for these mutants are predicted to produce truncated protein lacking the peptidase domain (Figure 14).

7.7.2 **CG12163 mutation as a fly model of Type B Kufs disease**

Since human patients with CTSF mutations and mice lacking Cathepsin F display neurological phenotypes in mid-life and premature death (Smith et al., 2013; Tang et al., 2006a), the lifespan of CG12163 mutant flies was assessed. Among the three mutants, only CG12163\textsuperscript{17} flies surprisingly displayed reduced survival while other mutants had similar lifespan as control flies (Figure 15A).

**Figure 15: Phenotypic characterisation of CG12163 mutants.** (A) Only one of the mutants showed reduced survival compared to controls. (B) Young 1-day old mutant flies did not display any difference in survival under oxidative stress conditions in 3% H\textsubscript{2}O\textsubscript{2}. (C) Old 25-
day old mutant flies are sensitive to oxidative stress, except CG1216318. (D) All mutants displayed an age-dependent decline in motor ability measured by negative geotaxis.

Since oxidative stress has been shown to influence models of neurodegeneration in flies, we asked if CG12163 has any role in resistance to oxidative stress. Briefly, we examined the survival of CG12163 mutant flies after acute exposure to the potent oxidizer hydrogen peroxide (H2O2). Young 1-day old grown on media supplemented with H2O2 at a final concentration of 3% did not show marked differences in survival compared to control flies as observed from the slope of the curves (Figure 15B). However, old mutant flies (25-day old) except CG1216318 showed increased sensitivity to oxidative stress (Figure 15C). This suggests that: (1) at least to an extent, CG12163 influences hypersensitivity to oxidative stress and; (2) the consequence of CG12163 mutations take effect in later stages of the fly life, consistent with late/adult-onset phenotypes observed in mammalian CTSF mutants (Smith et al., 2013; Tang et al., 2006a). We hope to account for the phenotypic differences among the 3 mutants by performing rescue experiments, and by including other mutants in future experiments to rule out off target effects. Notwithstanding this disparity, all three mutants displayed marked reduction in motor ability measured by the number of flies able to climb up to a specified height within a specified time (Figure 15D). In fact, the severity of the climbing defect increased with age. Taken together, CG12163 influences certain aspects of neuronal function in Drosophila. Brain vacuolisation is one of the typical signs of neuronal loss in Drosophila brain. In 30-day old flies, we observed slightly increased brain vacuoles compared to controls (Figure 16).
Figure 16: Adult brains of CG12163 mutants display slightly increased brain vacuoles. Black arrows indicate position of vacuoles.

One of the pathological features of NCL is the accumulation of autofluorescent granules in the brain tissues of patients due to accumulation of lipofuscin (Di Fabio et al., 2015; Smith et al., 2013). In a Drosophila NCL model carrying Cathepsin D mutation, autofluorescence could also be observed in adult brains (Kuronen et al., 2009; Myllykangas et al., 2005). We decided to examine larval wing discs of mutant flies and were surprised to find that they display tissue autofluorescence so early in development (Figure 17).
**Figure 17**: CG12163 larval wing discs display autofluorescence.

Since CG12163 was isolated as both a Vps25 and a Delta modifier, we wondered whether the mutants display any defect in Notch signalling. We probed mutant tissues with an antibody against the cleaved intracellular form of Notch, and did not observe any change in Notch activation (Figure 18). Thus, CG12163 mutation on its own does not influence Notch signalling in the Drosophila brain.
Figure 18: CG12163 mutant wing discs do not display changes in Notch cleavage, based on levels of Notch intracellular domain (NICD)

Cathepsin are lysosomal proteases and are important for lysosomal functionality. We therefore wondered if the CG12163 mutants have defective lysosomes. Using the dqBSA assay which measures the ability of the lysosome to cleave a fluorogenic substrate, we observed no gross changes in lysosomal function (Figure 19). This suggests either CG12163 mutations are not sufficient to induce lysosomal dysfunction or the changes in lysosomal function are beyond the sensitivity of our assay.
**Figure 19:** *CG12163* mutant larval wing discs do not display gross defects in lysosomal function, measured using the dqBSA assay.

### 7.7.3 *CG12163* loss causes p62 degradation and transcriptional upregulation of Atg8

*CG12163*, being the potential orthologue of *CTSF*, is predicted to be a lysosomal enzyme and studies have extensively linked the autophagy-lysosomal pathway with maintenance of neuronal health. Also, several lysosomal proteins have been shown to affect autophagy [reviewed in (Mrschtik and Ryan, 2015; Son et al., 2012)]. To check for autophagic dysfunction, we performed immunoblotting against p62 on brain extracts of *CG12163*-
deficient flies. p62 links ubiquitinated proteins to the autophagic machinery, leading to their degradation (Rogov et al., 2014). When autophagy is defective, p62 accumulates and this have been shown to be functionally important in several types of neuropathologies (Bartlett et al., 2011). Western blot analysis on adult heads revealed alteration in p62 levels. Rather than increased p62, which is almost a hallmark of several neurological diseases, we observed reduced levels of p62 across the three mutants (Figure 20A). This reduced level of p62 is post-transcriptional because we did not observe a change in p62 transcript levels (data not shown). Taken together, CG12163 loss might alter autophagic processes by influencing the p62 stability or by accelerating p62 clearance.

![Bar graph showing p62 protein levels in CG12163 mutant heads compared to control.](image)

**Figure 20: CG12163 mutant brains display alteration of autophagy.** (A) p62 protein levels are reduced in 30-day old adult mutant heads. (B) CG12163 mutant adult heads all display transcriptional upregulation of Atg8, but not Lamp1.

Transcription factor EB (TFEB) gene is a master regulator of lysosomal biogenesis and autophagy and its activity is critical for autophagic clearance by regulating expression of genes belonging to the Coordinated Lysosomal Expression and Regulation (CLEAR) network.
in mammals and flies (Bouché et al., 2016; Roczniak-Ferguson et al., 2012; Settembre et al., 2011, 2012; Tognon et al., 2016). One of the genes acting downstream of TFEB (Mitf in Drosophila) which is crucial for autophagosome formation and maturation is Atg8. By qPCR, we observed upregulation of Atg8 transcript levels in all three CG12163 deficient lines. In contrast, the lysosomal gene Lamp1, which is also a TFEB target is significantly upregulated only in one of the mutants (Figure 20B). These results indicate that loss of CG12163 induces an autophagic response in adult fly heads that is regulated at transcriptional level and might be at least partially independent of TFEB, since Lamp1 is not comparably elevated.
8 DISCUSSION

Here, we report the first in vivo genetic screen for ESCRT gene Vp25 in Drosophila, which allows systematic dissection of interacting genes. Our results support the idea that Vps25 and indeed ESCRT functions influence multiple cellular pathways. Importantly, some of the genes that we have identified as modifiers are not characterized, but have human homologues that have been associated with various diseases. Our results thus have the potential to uncover genes that play important roles in regulating the downstream effects of ESCRT, including but not limited to endosomal sorting and signal transduction; and this may be relevant in diseases such as some forms of cancer and neurodegeneration. Some of them have been previously associated to ESCRTs, but most others are not yet. This is highlighted by our identification of dop, the Drosophila homologue of mammalian MAST kinase genes, whose mutations have been linked with breast and other cancers; and CG12163, the Drosophila homologue of mammalian cathepsin F, whose mutations cause a rare form of neuronal ceroid lipofuscinosis in humans. The implications of our findings are detailed below.

8.1 Involvement of ESCRTs and endosomal sorting in a wide range of biological processes.

Consistent with our initial expectations, our results showed that Vps25 genetically interacts with key endosomal and trafficking genes such as Vps22/lsn, Vps24, Vps36, Chmp1, Syx7, Syx13, Rab8 and sec63. However, loss of one copy of these genes did not yield the same type of modification. We will attempt to explain the reasons that could account for this difference, focusing on ESCRT genes.

We expected that reducing the gene dosage of any ESCRT gene would enhance the Vps25RNAi phenotype, due to aggravation of the endosomal sorting defect. However, while Vps2 and Vps22/lsn acted as enhancers, Vps36 acted as a suppressor. Vps22 and Vps36
interact to form one lobe of the trilobar ESCRT-II complex and two Vps25 subunits make up the remaining complex (Hierro et al., 2004; Im and Hurley, 2008; Teo et al., 2004). Although Vps36 and Vps22 belong to the same complex, intrinsic structural differences between them might dictate specific roles they play in cargo sorting at the endosome, and the resulting biological outcomes. Indeed, there are examples that demonstrate that Vps36 are likely to possess specific functions, independent of the other two ESCRT-II subunits: (1) Although the observation that tissues entirely mutant for Vps22, Vps25 and Vps36 all display neoplastic phenotypes (with similar accumulation of ubiquitinated proteins and ectopic activation of Notch, JAK/STAT, JNK signalling pathways) does not support the existence of differences among these subunits (Herz et al., 2009; Woodfield et al., 2013a), analyses of mutant clones in mosaic Drosophila imaginal discs reveal that Vps36 differs from either Vps22 or Vps25 in terms of hyperplastic non-autonomous overgrowth of neighbouring wild type tissues, apoptosis and Notch signalling (Herz et al., 2009). While Vps22 and Vps25 clones induce non-autonomous overgrowth and ectopic Notch signalling, Vps36 clones do not (Herz et al., 2009; Thompson et al., 2005; Vaccari and Bilder, 2005); (2) In a study describing the role of ESCRT-II in localisation of the bicoid mRNA in Drosophila oocyte, vps36 but not the other subunits was shown to specifically bind the bicoid mRNA and regulate its localisation, thus playing a role likely independent of endosomal trafficking (Irion and St Johnston, 2007); (3) Vps36, by virtue of its GLUE domain (Hierro et al., 2004; Im and Hurley, 2008; Slagsvold et al., 2005; Teo et al., 2006) is able to specifically interact with specific cargoes like Smo of the Hedgehog signalling pathway (Yang et al., 2013). Although we have not directly tested these features of the ESCRT-II complex in our system, it is plausible that these might play a role in the phenotypic modifications we have observed. This observations might not only apply to vps36 alone, as other ESCRT-II subunits can perform specific functions independent of endosomal sorting (Ghoujal et al., 2012).
Gene ontology analysis and functional classification of modifying genes revealed that several biological processes might influence or be influenced by Vps25 function. Generally, our Vps25 interaction network suggest that endosomal sorting is a prominent function, relative to others because most of the modifiers that we identified are directly involved with trafficking, and ubiquitin-dependent MVB sorting is one of the pathways most significantly enriched in our gene lists. Other enriched processes or functional gene classes were as expected; signalling [reviewed in (Constam, 2009; Gonzalez-Gaitán and González-Gaitán, 2003; Sigismund et al., 2012)], apico-basal polarity (Lu and Bilder, 2005; Rodahl et al., 2009; Vaccari and Bilder, 2005, 2009); ubiquitination [reviewed in (Haglund and Dikic, 2012; Katzmann et al., 2001; Polo, 2012; Raiborg and Stenmark, 2009; Shields and Piper, 2011)]. Here, we will attempt, using the literature and synthesis, to focus on novel and unexpected interactions for which the link with ESCRTs is not immediately apparent.

8.1.1 Modification by cell division, DNA replication and repair genes

We have identified genes encoding regulators of cell division and DNA replication as modifiers of the Vps25RNAi phenotype. Some of these genes, such as Bub1, Mad1 and AurA function at the spindle assembly checkpoint (Chen, 2002; Emre et al., 2011; Krenn and Musacchio, 2015; Logarinho et al., 2004); Rcd5, functions in duplication of the centriole and also in transcription (Dobbelnaere et al., 2008; Raja et al., 2010); Chro, maintains microtubule spindle dynamics and chromosome structure (Ding et al., 2009; Rath et al., 2004, 2006); mgr, maintains the structure of spindle poles (Delgehyr et al., 2012; Gonzalez et al., 1998; González et al., 1988); ens, regulates microtubule growth and centrosome separation (Barlan et al., 2013; Gallaud et al., 2014; Sung et al., 2008); gwl, controls proper chromosome segregation (Archambault et al., 2007; Wang et al., 2013; Yu et al., 2004); cdc25, regulates cell cycle progression (Edgar and O’Farrell, 1989, 1990); Rfc4, regulates cell cycle checkpoint and DNA replication (Krause et al., 2001); Irbp/Ku70, is involved in non-
homologous end joining of DNA strand breaks and maintenance of telomere length in
*Drosophila* (Melnikova et al., 2005; Min et al., 2004); *mei-P22*, is required for meiotic
recombination during oogenesis (Liu et al., 2002a); *okr*, functions in the DNA repair pathway
(Ghabrial et al., 1998).

The fact that we identified cell division genes as modifiers is not entirely surprising,
given the body of evidences demonstrating the role of Drosophila ESCRT complexes in
cytokinesis; this however, has not been observed in mammalian cells. In fact, it is likely that
ESCRTs evolved as a machinery for cytokinesis (Field and Dacks, 2009). Although ESCRT-
II have been shown to be largely dispensable for the abscission part of the cytokinesis process
(McCullough et al., 2013), studies have revealed that ESCRT-II components Vps25 and
Vps36 also localise to the cytokinetic bridge and are responsible for recruiting ESCRT-III
components (Goliand et al., 2014). It is thus possible that some of the phenotypes associated
with ESCRT-II mutants or RNAi knockdown might result cell division defects. More
generally, endocytosis and trafficking play important roles at several stages of cell division
(Chen et al., 2012; McKay and Burgess, 2011). For example, the Rab5 GTPase regulates
nuclear envelope breakdown at mitotic entry and also regulates proper chromosome
alignment (Capalbo et al., 2011; Lanzetti, 2012), Rab11 regulates spindle alignment (Ai et
al., 2009; Zhang et al., 2008), the SNARE protein Snap29 promotes assembly of the
kinetochore (Morelli et al., 2016), syntaxin 16 is required for the accumulation of recycling
endosomes and recruitment of ESCRT machinery to the midbody at late telophase (Neto et
al., 2013). Conclusions that can be drawn here are: (1) our study further confirms the function
of endosomal sorting genes in cell division processes; (2) cell division defects might underlie
several defects associated with depletion of ESCRT components in *Drosophila* tissues; (3)
cell division aberrations might also contribute to human pathologies caused by either
defective endosomal sorting or mutations of endocytic genes.
There is not much known about the function of ESCRT proteins or endosomal sorting/trafficking in DNA repair and genome stability. In yeast, ESCRT subunits are required to prevent accelerated shortening of telomeres (Dieckmann et al., 2016), and ESCRT-III in mammalian cells protect against DNA damage by regulating nuclear envelope assembly (Vietri et al., 2015). There might be more indirect ways by which ESCRTs or endosomal sorting events might protect against DNA damage, such as: (1) regulation of autophagy – ESCRTs have been shown to play important roles in autphagic events (Rusten and Simonsen, 2008; Rusten et al., 2007), and defective autophagy causes increased DNA damage and chromosomal aberrations. Also, autophagy via ubiquitin signalling regulates the activity of DNA repair proteins (Feng and Klionsky, 2017; Wang et al., 2016; Xu et al., 2017); (2) by promoting proper cell division, ESCRT and endosomal sorting might contribute towards maintenance of genome stability (Bakhoum et al., 2017; Ganem and Pellman, 2012; Heijink et al., 2013); (3) We now know that ESCRTs and endosomal sorting control multiple signalling pathways, and this might indirectly regulate DNA damage/repair, for example, JAK/STAT signalling pathway activates ATM-mediated DNA damage response (Hong and Laimins, 2013; Rosen et al., 2010; Silver-Morse and Li, 2013) and Notch signalling negatively regulates DNA damage response (Adamowicz et al., 2016; Vermezovic et al., 2015). In addition, analysis of modifiers identifies the GO term ‘response to oxidative stress’ as being significantly enriched. It is still unclear the precise role of ESCRT in DNA damage response and this merits further investigation. Perhaps, genome instability contributes to some of the human pathologies like cancer and neurodegeneration that are caused by ESCRT mutations.

8.1.2 Modification by RNA processing genes
Since our screen is based on modification of an RNAi phenotype, we initially thought modifiers involved with RNA processing might reflect non-specific modification of the
Vps25RNAi phenotype rather than genetic interaction. Although we cannot completely rule this out, studies have demonstrated that ESCRT proteins directly regulate RNA processing. As previously discussed, one of the early evidences show that Vps36 determines correct localisation of bicoid mRNA in the Drosophila oocyte and this is dependent on the RNA-binding protein Staufen (Irion and St Johnston, 2007). Further studies have shown that ESCRTs control microRNA (miRNA) activity by associating with components RNA-induced silencing complex (RISC) (Gibbings et al., 2009), and ESCRT-II proteins bind Staufen and the HIV-1 Gag protein, and this interaction is responsible for the genomic trafficking of HIV-1 viral RNA (Ghoujal et al., 2012). The contribution of ESCRTs on RNA metabolism in physiological or pathological conditions is unknown but it might include direct effects on gene expression. Indeed, prior to the discovery of their role in regulation of membrane events, mammalian ESCRT-II proteins were reported to form a complex with the RNA polymerase II elongation factor ELL in order to exert transcriptional control activity (Kamura et al., 2001; Schmidt et al., 1999).

8.2 A strategy to identify novel regulators of cell growth associated to Notch and endosomes

We used a Delta-induced eye overgrowth system as a secondary screen to identify those Vps25 modifiers that also influence tissue growth. The rationale was to uncover those modifiers that might be relevant for the role of Vps25 in tumorigenesis, and we isolated 43 genes that modify both Vps25 downregulation and Delta overexpression. Overexpression of the Notch ligand Delta under the eye-specific promoter (eyGAL4-Dl) causes a non-metastatic overproliferation and has been used to identify genes that influence tumour growth in Drosophila (Ferres-Marco et al., 2006; Herz et al., 2010; Miles et al., 2011). Some of our eyGAL4-Dl modifiers like eyg, toe, lqf are already known to be components of Notch
signalling pathway (Dominguez et al., 2004; Overstreet et al., 2004; Tian et al., 2004; Zhu et al., 2017). Others like Abl, disp, hyx, ImpL2 and bnl function in other signalling pathways, highlighting the emerging concept that the endosome acts as a nexus for intracellular signal transduction (Bader et al., 2013; Burke et al., 1999; Mosimann et al., 2006; Singh et al., 2010; Sutherland et al., 1996). We also have found genes that encode V-ATPase subunits, cell division regulators and several uncharacterised genes as modifiers of eye overgrowth. Taken together, the modifiers identified with the eyGAL4-Dl secondary screen either directly affect Delta/Notch signalling or may affect downstream pathways that are required for tissue growth.

### 8.3 Drosophila dop might control Notch signalling by regulating Delta expression

One of the previously characterized tissue growth modifiers is dop, the Drosophila homologue of mammalian MAST kinase genes. To be best of our knowledge, this is the first time dop has been linked with tissue growth. dop has previously been shown to regulate cellularisation of the Drosophila embryo during development (Galewsky and Schulz, 1992; Hain et al., 2014). In our hands, loss or reduced dop expression enhances the Delta-mediated overgrowth, suggesting that dop activity is antagonistic to the excessive growth caused by ectopic Notch activity. In fact, we have observed, also for the first time, that reduced dop levels cause an increased expression of Delta mRNA, and this also correlates with the increase in expression of the Notch target, E(spl)mß. This seems to be specific because we did not observe a change in the expression of Notch. Since regulation of Delta expression and Notch signalling is important during development, it will be interesting to understand if dop might influence cell fate and lateral inhibition during development. Since proneural proteins positively regulate Delta expression (Bertrand et al., 2002), perhaps, dop might influence the activity of proneural factors, perhaps by phosphorylation. The exact mechanism demands further study. Alternatively, mammalian MAST kinases have been found to associate with,
and control the stability and cellular localisation of the tumour suppressor protein PTEN (Terrien et al., 2012; Valiente et al., 2005). Thus, it will be interesting to test in the future whether *dop* regulates growth independently of Notch signalling by acting on the PTEN/PI3K pathway.

### 8.4 Mammalian MAST2 does not influence Notch signalling but might regulate other pathways

We find that knockdown of MAST2 in MCF10A cell lines, which endogenously express most Notch pathway proteins, does not change the expression of Notch-related genes. We selected MAST2 because it is the founding member of the MAST kinase family (Walden and Cowan, 1993) and the most expressed in MCF10A cells. Our negative results suggest that: (1) the role of *Drosophila dop* in Notch signalling is not conserved in mammals; (2) MAST kinase proteins other than or in addition to MAST2 might be performing this role. We will need to knockdown the other MAST genes both individually and simultaneously to identify which of these possibilities is the case.

In human breast cancers, translocations involving *MAST1* and *MAST2* have been identified (Robinson et al., 2011). However, little is known about the biological role of MAST kinases. MAST2 (also known as MAST205) was initially identified in microtubules of the spermatid, where it was found to play a role in sperm maturation (Walden and Cowan, 1993; Walden and Millette, 1996). MAST2 also regulates the activity of ion channels (Ren et al., 2013; Wang et al., 2006). Most of these findings do not point to an obvious explanation of how MAST kinase activity or inactivity might cause or promote tumour development. In contrast, the discovery that MAST2 binding to PTEN enhances its stability, promotes phosphorylation and nuclear relocalisation or retention of PTEN (Terrien et al., 2012; Valiente et al., 2005), suggest how MAST kinases might influence cell growth during development.
We have attempted to understand how MAST2 might promote tumorigenesis by analysing the expression of genes whose levels are known to be important for tumour development. The transcripts of FN1, MMP1 and MMP9 were observed to be upregulated upon MAST2 knockdown in breast epithelial cells MCF10A. What these 3 genes have in common is that they all are transcriptional targets of the nuclear factor kappa B (NF-κB) signalling pathway (He, 1996; Lee et al., 2002; Norton et al., 2004; Vincenti et al., 1998). Briefly, the NF-κB pathway is activated by the binding of a ligand to a cell surface receptor (e.g., tumor necrosis factor-receptor (TNF-R) or a Toll-like receptor), which leads to recruitment, activation and relocalisation of several complexes. This culminates in the nuclear translocation of active NF-κB complexes where they induce gene expression, either alone or in combination with other transcription factors (Gilmore, 2006). This pathway have been shown to be play a key roles in immunity, inflammation, development and importantly in cancer (Karin, 2006). Interestingly, MAST3 has been shown to be either positively inversely correlated with increased NF-κB-driven inflammation during inflammatory bowel diseases (Labbé et al., 2008, 2012; Majumdar et al., 2017). The strongest evidence comes from the fact that by binding to TRAF6 (an E3 ubiquitin ligase), MAST2 might negatively regulate NF-κB activity (Funakoshi-Tago et al., 2009; Xiong et al., 2004). Taken together, MAST2 or MAST kinases in general, might influence cancer development and progression by regulating the NF-κB signalling pathway. Although, Robinson and colleagues have reported that the genetic alterations of MAST1 and MAST2 in breast cancers lead to their transcriptional upregulation (Robinson et al., 2011), bioinformatics analysis of cancer databases shows that several other cancers carry mutations in MAST kinase genes. Perhaps it is misregulation, rather than downregulation, of MAST kinases that promote tumour development. Also by regulating FN1, MMP1 and MMP9 expression, MAST kinases might
influence epithelial-to-mesenchymal transition, and invasiveness during tumour development (Foda and Zucker, 2001; Lamouille et al., 2014).

8.5 Insights into human type B Kufs disease derived from the Drosophila model

A number of Vps25 and Delta modifiers were uncharacterized genes. One of these is CG12163 is the putative homologue of mammalian lysosomal enzyme cathepsin F. Mutations in human cathepsin-F cause an adult-onset form of neuronal ceroid lipofuscinosis (NCL) called type B Kufs disease (Smith et al., 2013; Tang et al., 2006b; van der Zee et al., 2016). Thus, we decided to use Drosophila to study CG12163 function in vivo and possibly establish for the first time a fly model of type B Kufs disease. Using CRISPR/Cas9, we generated knock-out lines with indels in the peptidase domain, the active part of the enzyme. In fact, in vitro evidences indicated that the human mutations impede the peptidase activity of the protein (Peters et al., 2015). Although, it is not currently known if the human mutations affect transcript levels in our fly mutants we observed almost complete loss of CG12163 transcript in our mutant lines, suggesting that also human mutations might be subjected to non-sense mediated RNA decay. NCLs are caused by accumulation of lipopigments in nerve cells leading to atrophy and patients with this form of NCL present difficulty coordinating voluntary movements (ataxia), speech difficulties, dementia and/or psychotic behaviour and premature death (Smith et al., 2013; Tang et al., 2006a; van der Zee et al., 2016). The fact that CG12163 is expressed predominantly in the fly head suggests that it may be relevant as a human model of the disease. Typical neurodegenerative phenotypes in Drosophila consist of reduced lifespan, locomotor defects, brain vacuolisation and sometimes increased sensitivity to oxidative stress (Sang and Jackson, 2005); a more specific NCL phenotype is the accumulation of lipofuscin. Although not all the three mutant lines displayed these phenotypes, at least 2 out of 3 mutant lines that we analysed displayed reduced locomotor
activity assayed by negative geotaxis, slightly increased brain vacuolisation compared to control, tissue autofluorescence and hypersensitivity to oxidative stress. Exactly why differences exist between the three mutants that we have studied is currently unclear. Whether this is due to off target effects of CRISPR or whether it reflects intrinsic diversity among the mutations will be a focus of future analysis. Indeed, it is possible that the stability or nature of the proteins produced from the residual mutant transcripts differ; should this be the case, this still does not account for why some phenotypes are common among all mutant lines and others are not. Despite this, our mutants recapitulate several aspects of the human disease suggesting that *Drosophila* may be a good model to understand the Type B Kufs pathogenesis.

### 8.6 NCLs and autophagy

Results of the dQ-BSA assay which measures digestive ability of the lysosome (Vázquez and Colombo, 2009) suggest that there is no marked change in the general ability of the lysosome to degrade substrates; this is reasonable because loss of one cathepsin might not be sufficient to observe a significant change, unless the enzyme has a broad-spectrum substrate. Thus, it would be interesting to assay in the future cleavage with more specific substrates. Indeed, in other forms of NCL due to loss of Cathepsins, the lysosomal defects can be quite specific (Holopainen et al., 2001; Koike et al., 2000). Because autophagy and lysosomal function are highly coordinated (Settembre et al., 2011) , we also assessed how autophagy functions in *CG12163* mutants. Autophagy has not been studied in the context of Type B Kufs disease but it might be very important for pathogenesis.

Indeed, increase in protein levels of the autophagy adapter p62, due to impaired autophagy is often found in aging brain and is observed in *Drosophila* models of neurodegeneration, as well as in autophagy gene mutants (Hara et al., 2006; Komatsu et al.,
Consistent with the possibility that type B Kufs disease involve alteration of autophagy, we found changes in p62 expression in \textit{CG12163} mutants. Rather than increase, we found reduction compared to control, suggesting that autophagy is somehow accelerated in \textit{CG12163} mutants. Two of the conditions that promotes autophagy and p62 reduction are oxidative stress and hypoxia (Pursiheimo et al., 2009; Small et al., 2014). \textit{CG12163} mutants are likely under oxidative stress, as evidenced by their hypersensitivity to hydrogen peroxide. Increased oxidative stress might contribute towards accelerated autophagy in mutant brains. Remarkably, hypoxia plays a role in the development of Alzheimer’s disease, but whether NCL patients present hypoxic brain tissues is not known. We envisage that either oxidative stress or hypoxia in \textit{CG12163} mutants might lead to excessive autophagy. Excessive autophagic activity has been proposed to lead to autophagic stress, imbalanced autophagic flux and eventual cell death of neurons (Lee, 2012). Further evidence of autophagic stress is our finding that transcription of the autophagy gene \textit{Atg8} is increased in \textit{CG12163} mutant brains. However, our results suggest that the autophagic changes are likely a consequence, rather than a cause of the neuronal phenotypes observed in \textit{CG12163} mutant brains. It will be interesting to examine brain tissues of NCL patients to assess whether indeed they are subjected to hypoxic or oxidative stress, and to investigate the role of autophagy in the development of NCL disease.
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## 10 SUPPLEMENTARY DATA

Table S1: List of genes identified in the \( \text{Vps25RNAi} \) primary screen

<table>
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<th>Suppressors</th>
<th>Enhancers</th>
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<td>CG10083</td>
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<tr>
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<td>Rpn6</td>
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