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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 NFκB 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

Complex	Function	Evolutionary origin	Yeast	*Drosophila*	Human
ESCRT-0	Cargo recognition	Opisthokonta	Vps27	Hrs	HRS
			Hse1	Stam	STAM1, STAM2
ESCRT-I	Upstream adapter	Eukaryotes	Vps23	Tsg101	TSG101
			Vps37		VPS37A, B, C, D
			Mvb12	Mvb12	MVB12A, MVB12B
			Vps28	Vps28	VPS28
ESCRT-II	Bridging adapter		Vps36	Vps36	EAP45
			Vs22(Snf8)	Vps22	EAP30
			Vps25	Vps25	EAP20
ESCRT-III	Membrane remodeling/ filament	Archaea	Vps2	Vps2	CHMP2A, B
			Vps24	Vps24	CHMP3
			Vps32 (Snf7)	Vps32 (Shrub)	CHMP4A, B, C
			Vps20	Vps20	VPS20/CHMP6
			Vps46 (Did2)	Chmp1	CHMP1A, B
			Vps60 (Chm5)	Chmp5	CHMP5
Vps4 –Vta1	Membrane remodeling/ ATPase		Vps4	Vps4	VPS4A, B (SKD1, 2)
			Vta1	CG7967	VTA1 (LIP5)
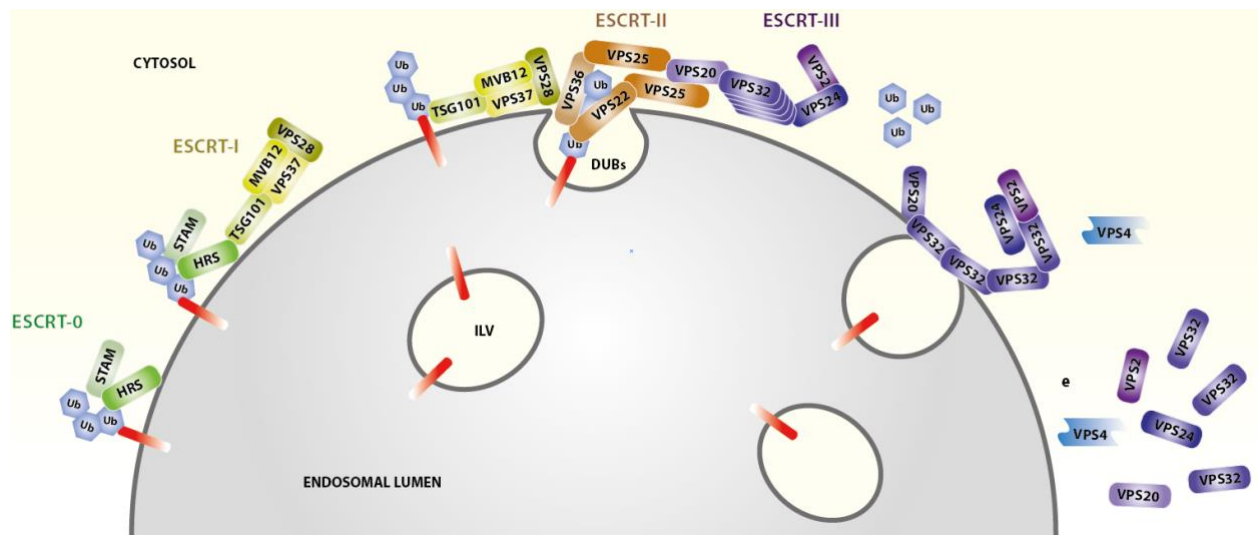
Figure 1: The ESCRTs in MVB biogenesis and receptor sorting. ESCRT-0, -I and -II interact with ubiquitinated cargo through their ubiquitin-binding domains, and sort them into intraluminal vesicles (ILVs). ESCRT-II recruits ESCRT-III to pinch/sever the ILV from the limiting MVB membrane. ESCRT-III polymeric 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).
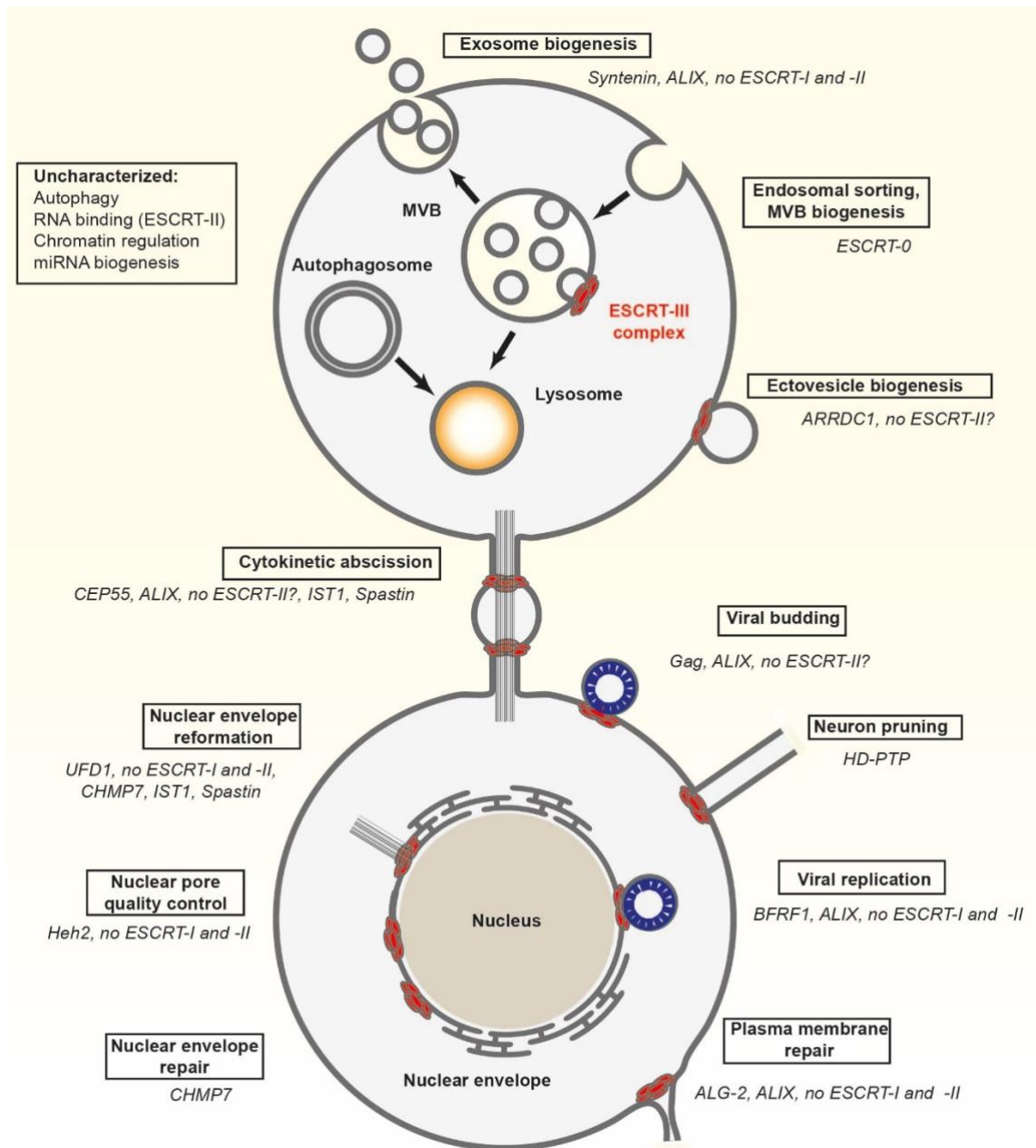
Figure 2: Summary of ESCRT-dependent processes. In addition to the well-known role of ESCRTs in receptor sorting and MVB biogenesis, several processes have been shown to require the function of ESCRT proteins. Specific factors required for each process are written in italics. (Alfred and Vaccari, 2016).

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 γ-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)].
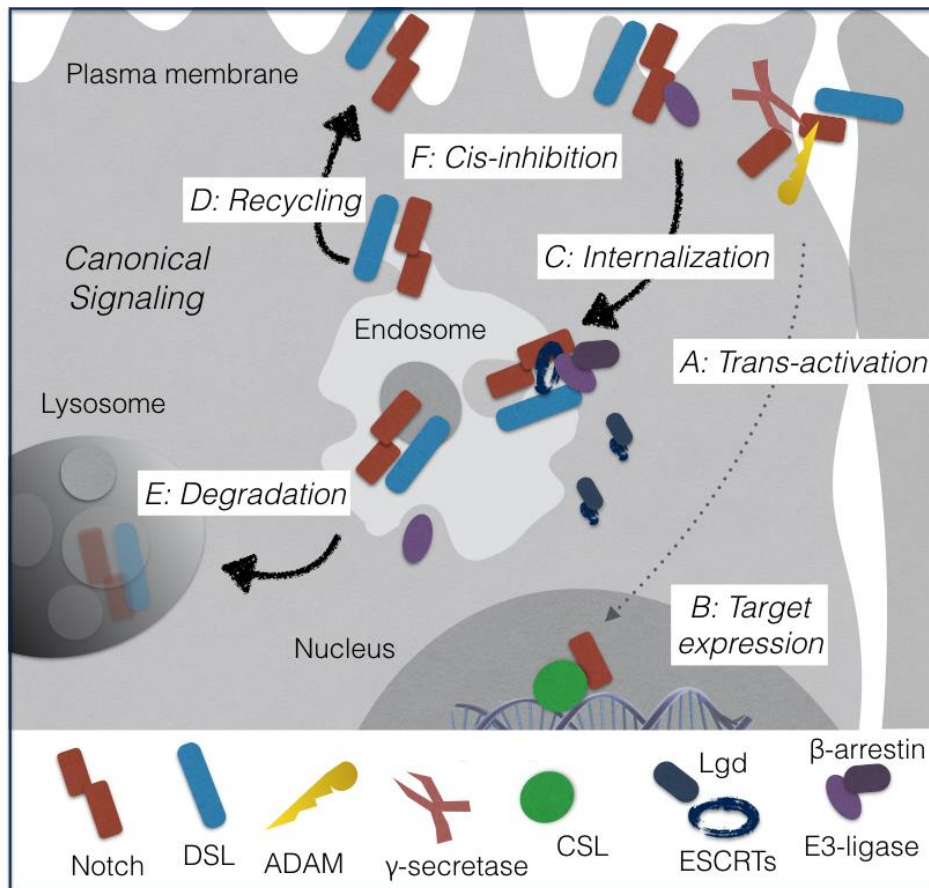
Figure 3*:* **Canonical Notch Signalling Pathway**. (A) Notch is cleaved to NICD by γ-secretase at the plasma membrane, or en-route to endosomes, upon trans-activation by a DSL ligand expressed in signal-sending cells. Extracellular cleavage by ADAM metallo-proteases is a prerequisite for γ- secretase 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 vescicles 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.,

1994; Diederich et al., 1994; Matsuno et al., 1995; Xu and Artavanis-Tsakonas, 1990). Indeed, ectopic activation of Notch signalling could be achieved by dx overexpression in *Drosophila* wing margin cells that lacked both Delta (Dl) 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 □-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.

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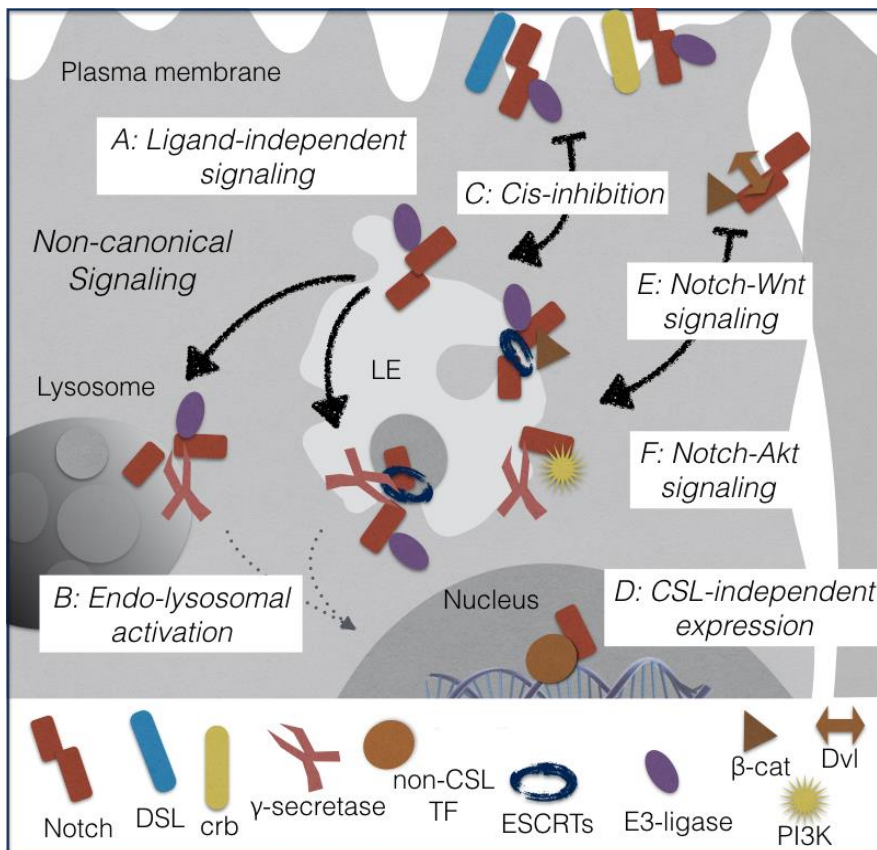
Figure 4: Non-canonical Notch signalling. (A) A pool of Notch can be activated in a ligand-independent fashion (B) Such pool of internalized Notch can be saved from endosomal sorting towards degradation by the activity of the E3 –ligase dx, and cleaved at the endosome, or upon fusion with the lysosome. 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, NFkβ 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 suggests 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 of ESCRT-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).

<u>Objectives</u>

    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/, Zhang Lab, MIT). 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' CTTCGACCTGGAACTTCTCCATGC 3'
	R:	5' AAACGCATGGAGAAGTTCCAGGTC 3'
CG12163	F:	5' CTTCGTGAAGAATCAGGGATCCTG 3'
	R:	5' AAACCAGGATCCCTGATTCTTCAC 3'
CG3764	F:	5' CTTCGCACAGCAGTACTTTCGTAC 3'
	R:	5' AAACGTACGAAAGTACTGCTGTGC 5'7
CG10147	F:	5' CTTCGGGCATGCGGTTTGTCTGCG 3'
	R:	3' AAACCGCAGACAAACCGCATGCCC 5'
Mkrn1	F:	5' CTTCGCTTGGAGTGCATTCGCACA 3'
	R:	5' AAACTGTGCGAATGCACTCCAAGC 3'
CG32113	F:	5' CTTCGTGGGTGCTGAACACGTATC 3'

R: 5' AAACGATACGTGTTCACGACCCAC 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 CO_2 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% CO_2 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% paraformaldeyde (PFA) diluted in H_2O 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 *Vps25^{A3}* 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).
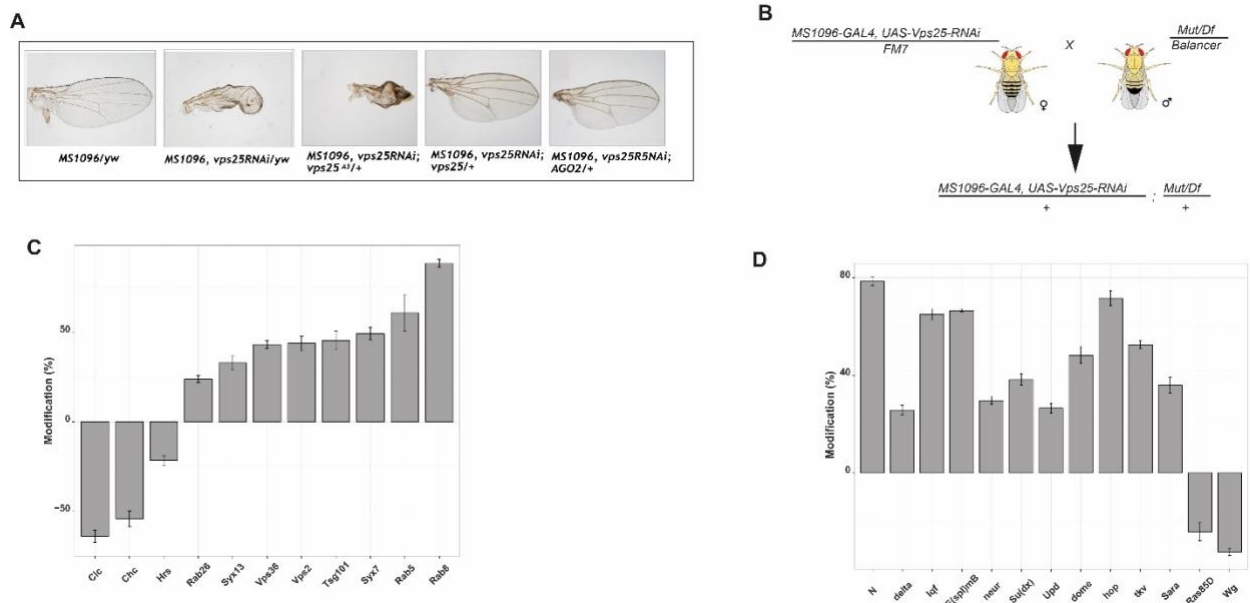
Figure 5: *Vps25* **knockdown causes a small wing phenotype sensitive to gene dosage**. **(A)** The small wing phenotype is caused by specific reduction of *Vps25* levels via RNAi. **(B)** Scheme showing the crossing strategy used for the screening. **(C)** Mutant alleles of trafficking genes modify 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 *Vps25* mutants, reduction of *Vps25* leads to overactivation of JAK/STAT signalling. The TGF-β/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-β signalling pathway contributes towards the *Vps25RNAi* phenotype. On the other hand, we unexpectedly observed that components of the RAS/RAF/MAPK and Wnt/Wg pathways enhance the *Vps25RNAi* phenotype (Figure 5D). This contrasts with results showing Ras and Wg activation in *Vps25* mutant tissues (Thompson et al., 2005; Vaccari and Bilder, 2005)

      Modification by trafficking components suggest that the use of the *Vps25* 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 *Vps25* null mutant tissue, Notch is ectopically activated *Vps25RNAi* wings. Finally, the fact that the *Vps25RNAi* wings are reduced in size, despite activation of proliferative Notch and JAK/STAT signalling, strongly suggest that, as in *Vps25* null mutant tissue, apoptosis is active in *Vps25RNAi* wings.
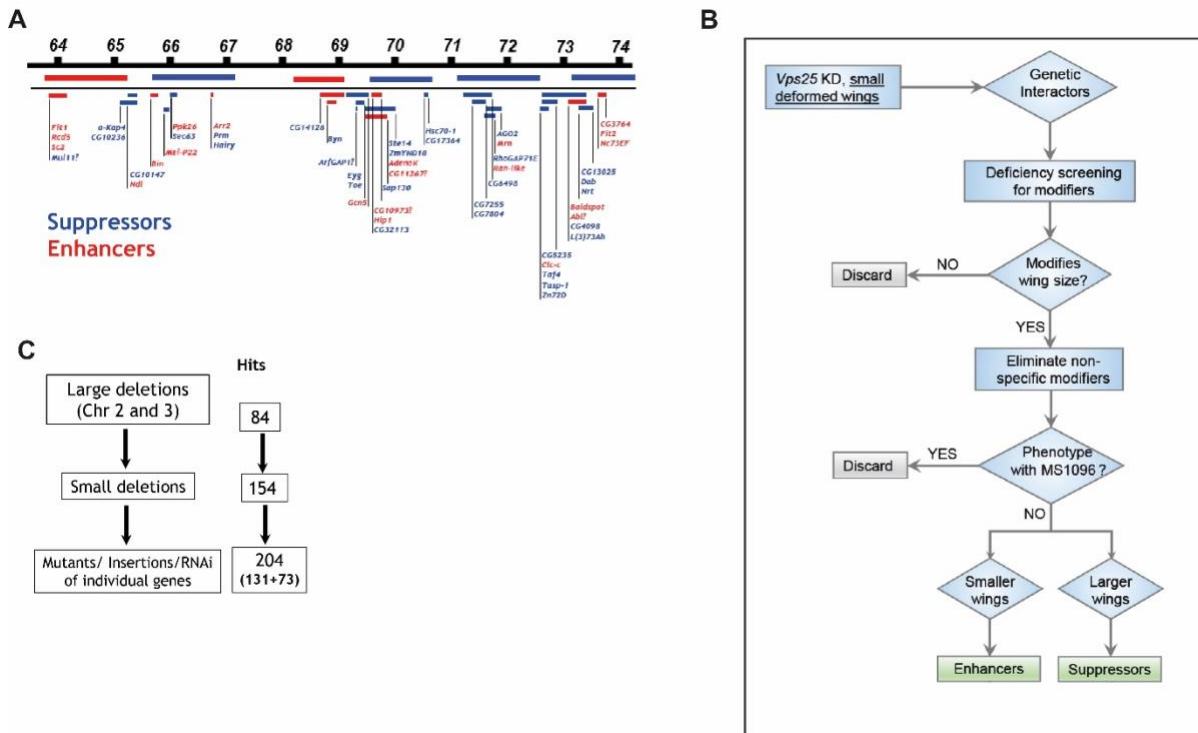
Figure 6: **Breakdown of screening strategy.** **(A)** We ued a deficiency screening approach to isolate modifiers, starting with larger defieicies, to smaller deficiencies to individual loci. The image shows a section of chromsome 3R. **(B)** Workflow of the screening approach. **(C)** Breakdown of 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 a 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).
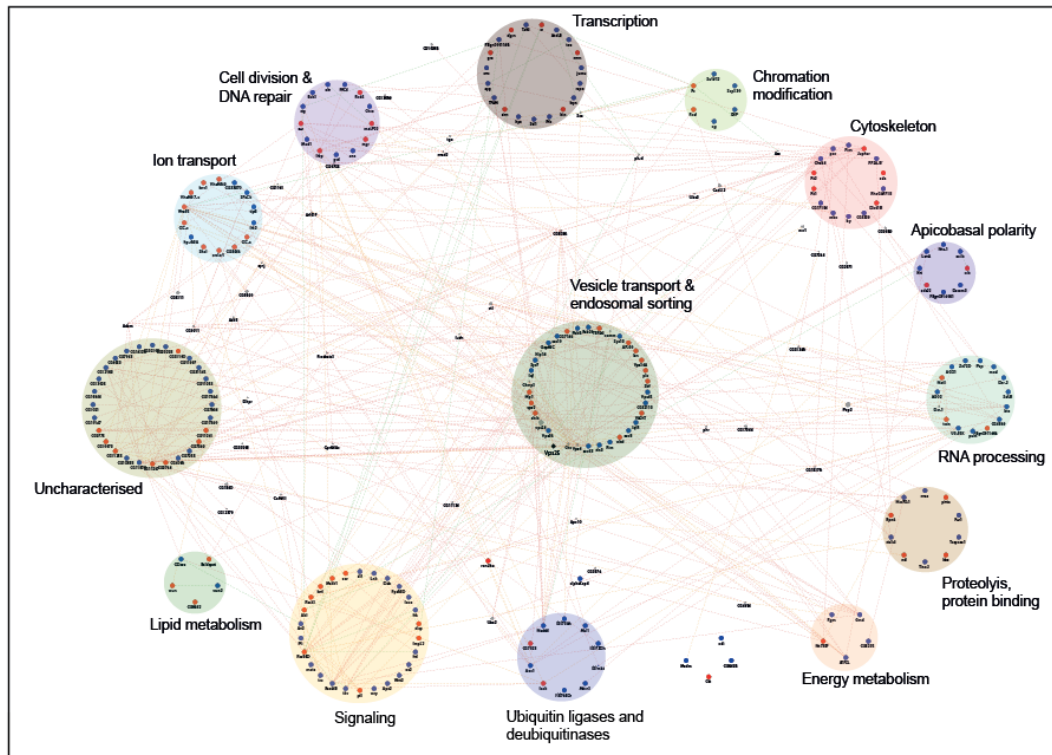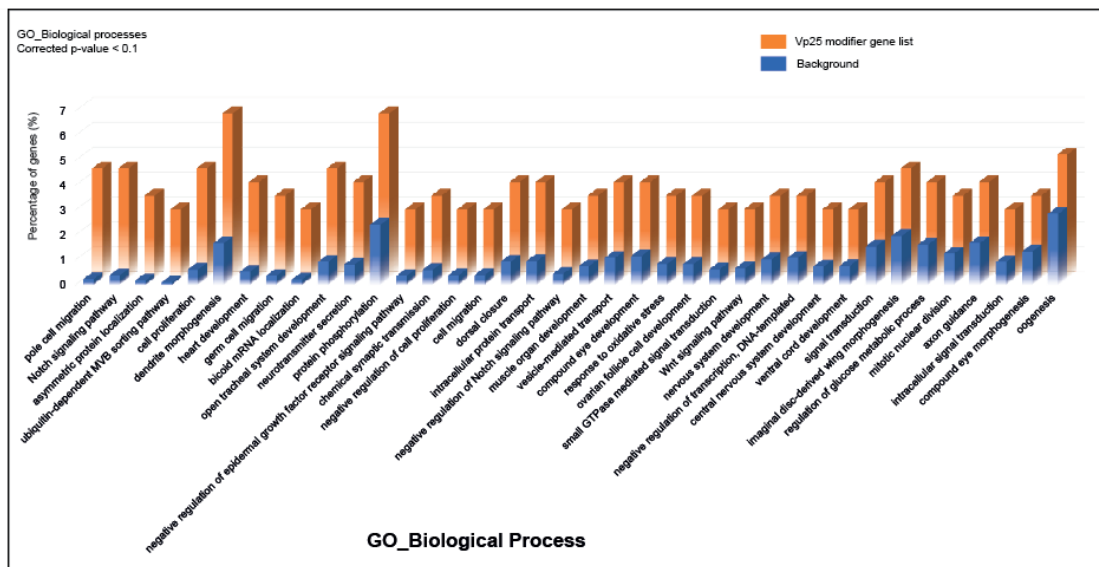
Figure 7: **Analysis of *Vps25* interactors**.(A) Network showing the categories of genes identified in the primary screen. (B) Gene Ontology analysis showing biological processes that are enriched.

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-Marco 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).
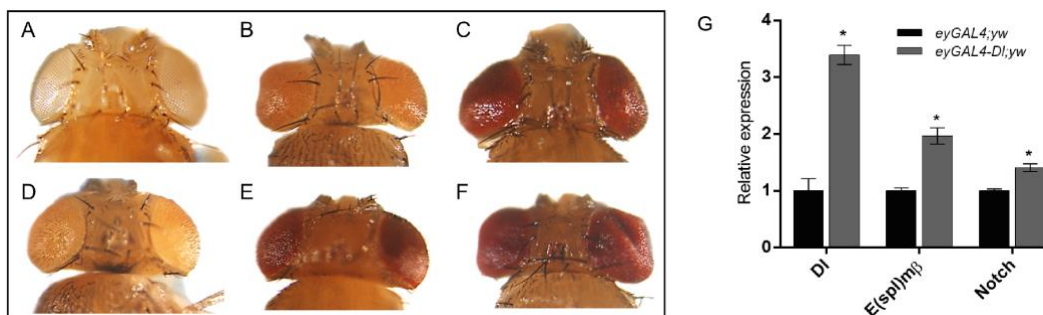
Figure 8: Delta overexpression in the Drosophila eye causes overgrowth by activating Notch signalling. (A) wild type (B) *eyGAL4-Delta*, eye overgrowth induced by *Delta* overexpression. Mutant alleles of *Delta* (C), *presenilin* (D) and *eyegone* (E) in the *eyGAL4-Delta* background suppress the eye 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)

	eyGAL-Delta **Suppressors**	*eyGAL-Delta* **Enhancers**
***Vps25RNAi* Suppressors**	• *CG17364* • *CG17360* • *lqf* • *hh* • *eyg* • *pum* • *Taf4* • *Aos1* • *sec63* • *CG11253* • *Pinta* • *RhoGAP71E* • *CG12163* • *toe* • *slif*	• *CG32109* • *CG6498/dop* • *cnc* • *l(3)76BDr* • *Hsc70-1* • *Pep* • *Nedd4* • *hyx* • *CG10147* • *Trc* • *Byn* • *CG32113*
***Vps25RNAi* Enhancers**	• *CG3764* • *disp* • *sds22* • *CG31100* • *bnl* • *ImpL2*	• *vhaM9.7-c* • *Arfip* • *vhaM8.9* • *bin* • *CG7369* • *Sup12-46* • *Rcd5* • *aur* • *Abl* • *ran-like*

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^{JF02778}* and *dop^{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 $29\pm0.04\%$ and $75\pm0.05\%$ 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.
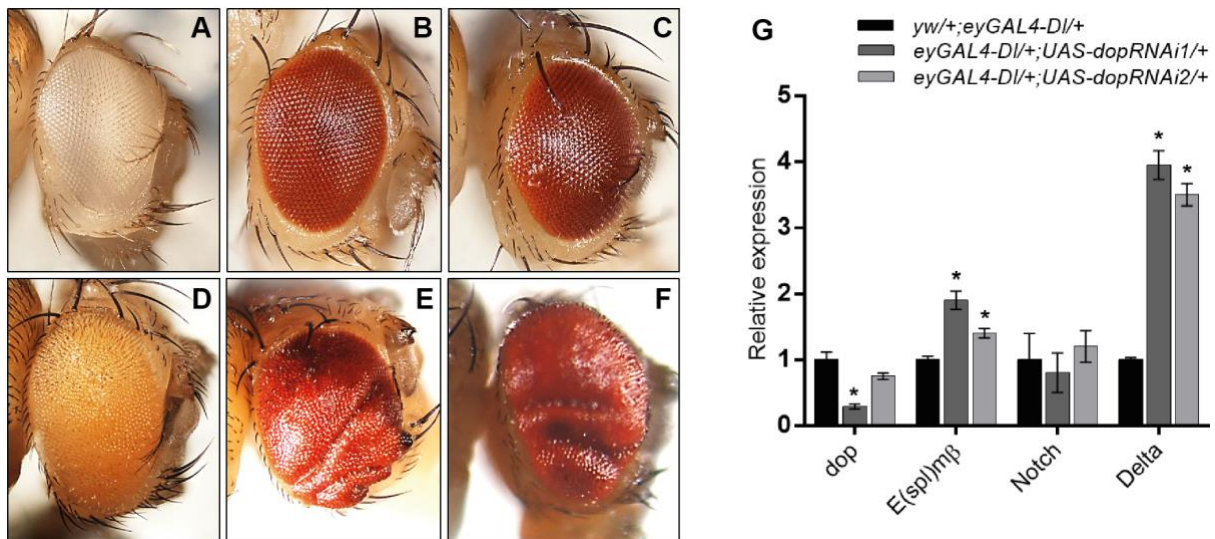
Figure 8: Reduction of dop enhances Delta-mediated eye overgrowth by upregulating *Delta* expression. Knockdown of dop in the *Drosophila* eye using two RNAi lines (B, C) has no effect compared to wild type eye (A). However, dop knockdown in the *eyGAL4, Delta* background enhances the eye overgrowth (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)mβ* negatively correlates with the levels of *dop* mRNA ($r^2 = -0.989$, using the R cor function). This increase in *E(spl)mβ* 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 *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)mβ independently of Notch signalling. We have checked for changes in the transcript levels of *Notch* and *Delta*: reduced *dop* levels in the *eyGAL4-Delta* background does not alter *Notch* expression but significantly increased the expression of its ligand, *Delta* (Figure 8A-G). We already know that the *eyGAL4-Delta* system is sensitive to levels of *Delta*; therefore, the upregulation of *Delta* might be responsible for the enhanced growth of *eyGAL4-Delta* eye tissue by dop downregulation.

     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 *dop¹*, 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.
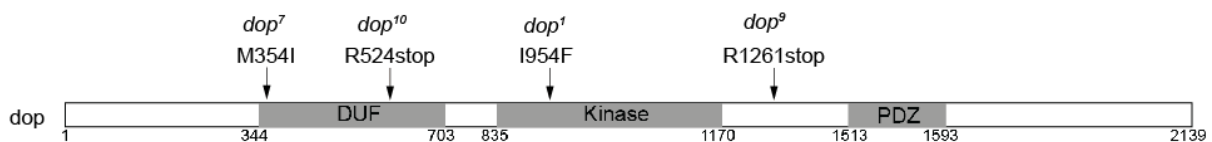
Figure 9: Location of *dop* mutations (Hain et al., 2014)
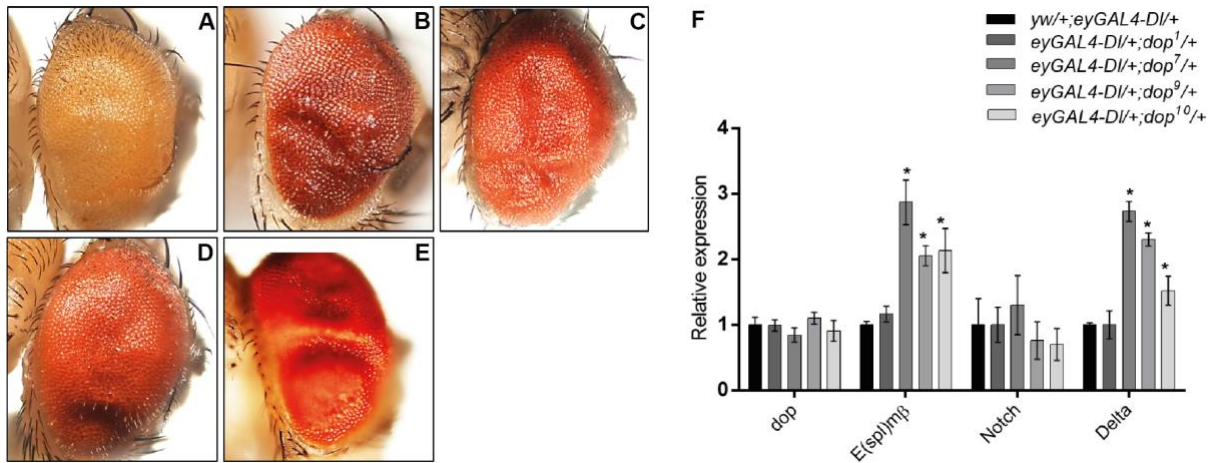
Figure 10: Mutant dop alleles enhance Delta-mediated eye overgrowth. Mutant *dop* alleles enhance Delta-mediated eye overgrowth. (A) *eyGAL4, Delta* (B) e*yGAL4, Delta/+; dop^1/+* (C) *eyGAL4, Delta/+; dop^7/+* (D) *eyGAL4, Delta/+; dop^9/+* (E) *eyGAL4, Delta/+; dop^{10}/+*

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).
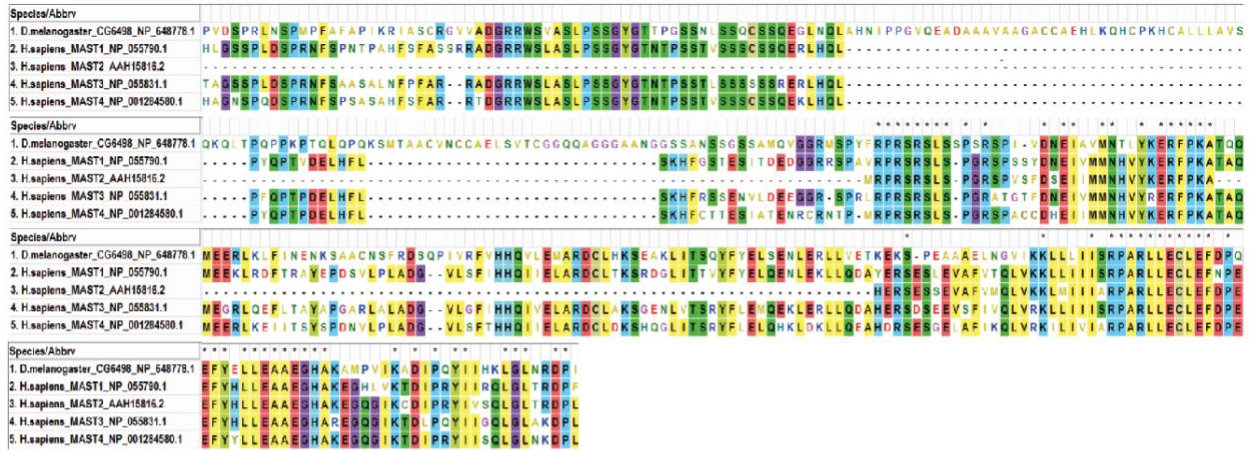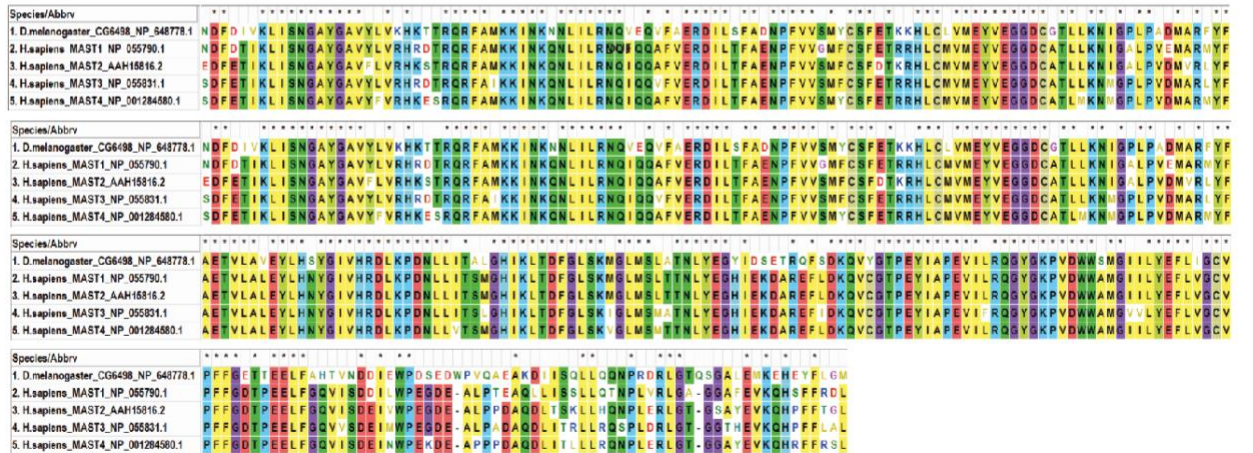
Figure 11: Homology alignment of dop to mammalian MAST kinases 1-4 across the three domains

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.
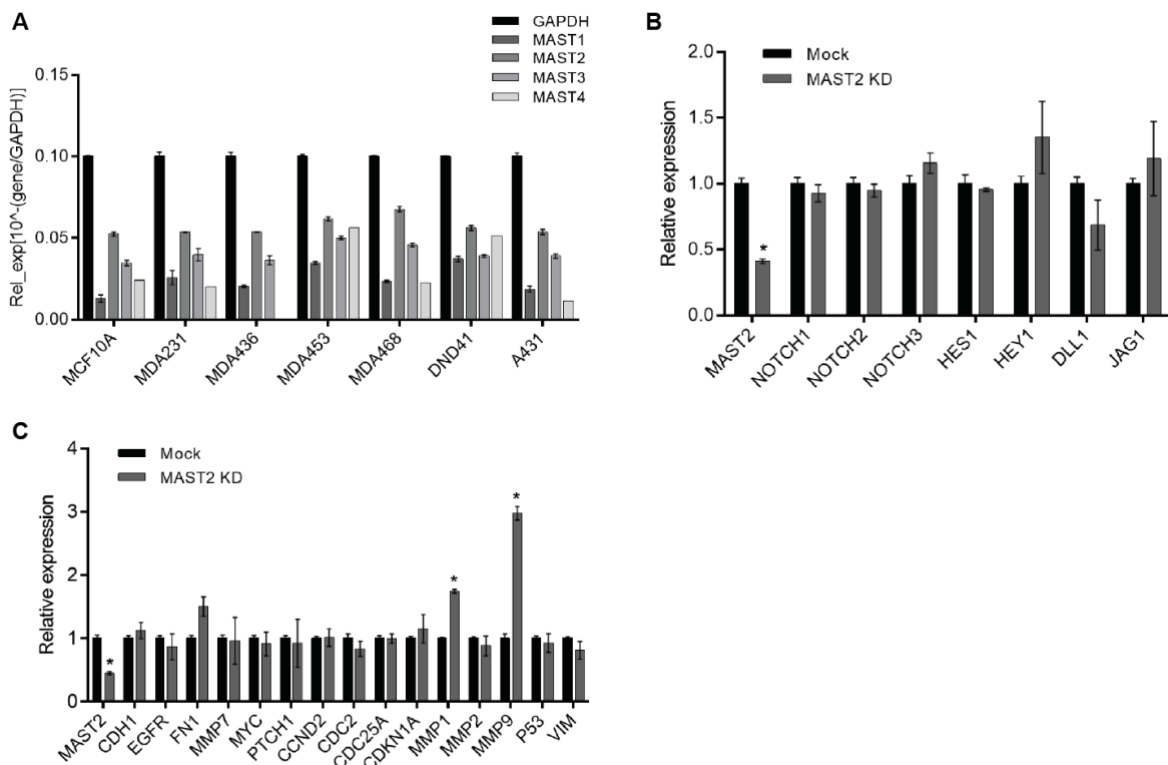
Figure 12: Analysis of MAST expression across cell lines, and effect of knockdown in MCF10A cells (A) Expression profiling of MAST2 expression across human cell lines reveal that MAST2 is the most expressed family of the MAST kinases. **(B)** MAST2 knockdown in MCF10A does not alter the expression of 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 an 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-Dl* 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/, 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.

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 *Mkrn1* 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 Mkrn1 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.
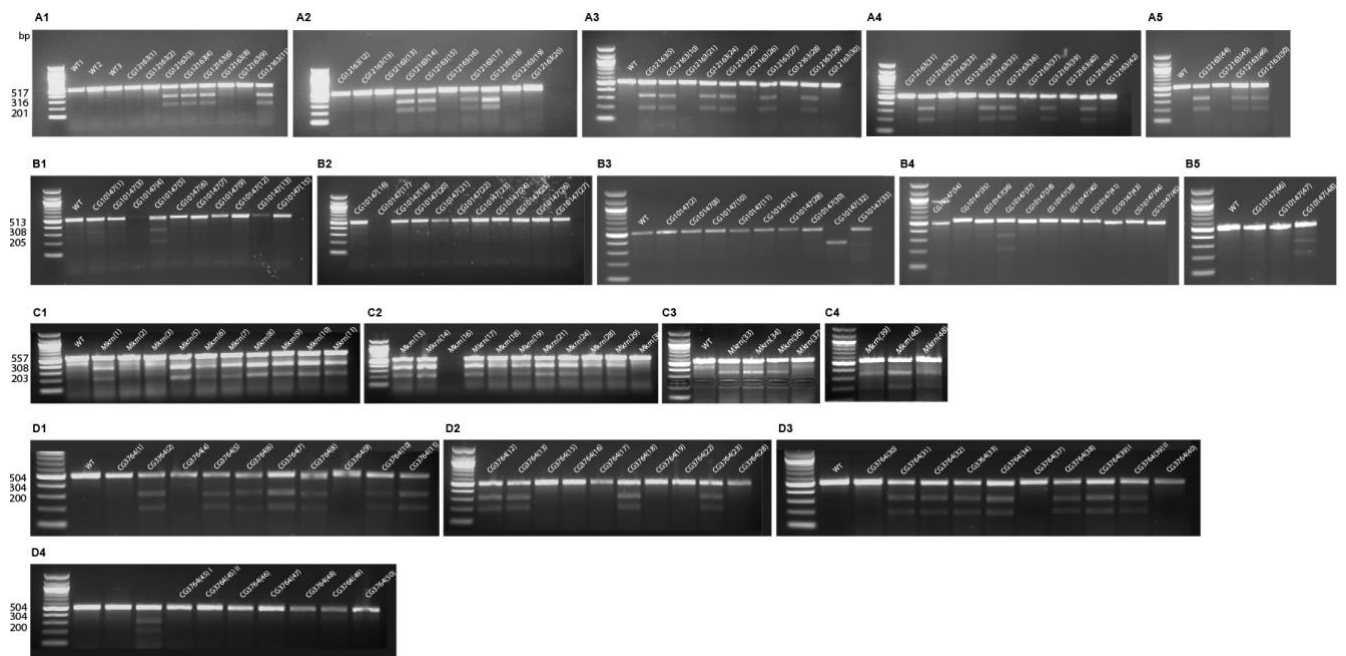
Figure 13: T7 endonuclease assay to identify indel mutants generated by CRISPR/Cas9.
Presence of triple bands per lane suggests the presence of indels greater than 3 bases.

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*.
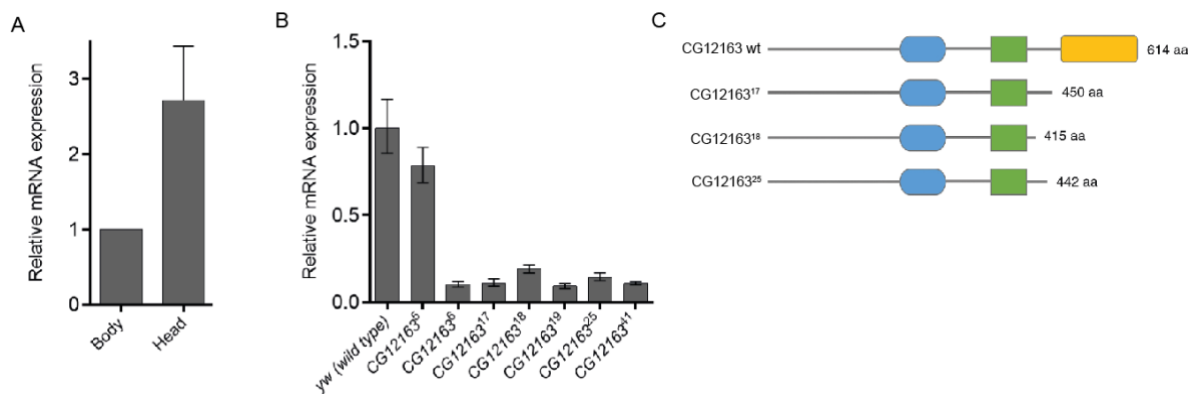
Figure 14: Description of *CG12163* mutants. (A) *CG12163* is predominantly expressed in the adult fly head compared to the rest of the body. (B) Most of the mutations cause reduction in *CG12163* transcript levels possibly due to mRNA decay. (C) *CG12163[17]*, *CG12163[18]*, *CG12163[25]* mutations 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 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[17]*, *CG12163[18]* and *CG12163[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 a 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[17]* flies surprisingly displayed reduced survival while other mutants had similar lifespan as control flies (Figure 15A).

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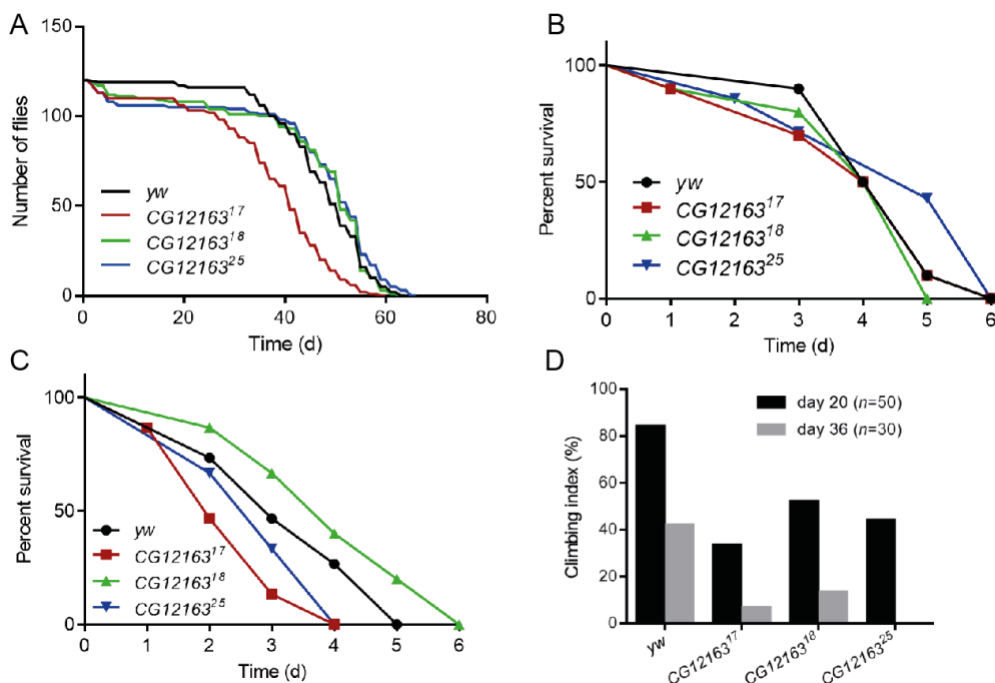
Figure 15: Phenotypic characterisation of *CG12163* mutants. (A) Only one of the mutants showed reduced survival compared to controls. (B) Young 1-day old mutant flies did not display any difference in survival under oxidative stress conditions in 3% H_2O_2. (C) Old 25-

day old mutant flies are sensitive to oxidative stress, except *CG12163*[18]. (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 (H_2O_2). 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 *CG12163*[18] 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).
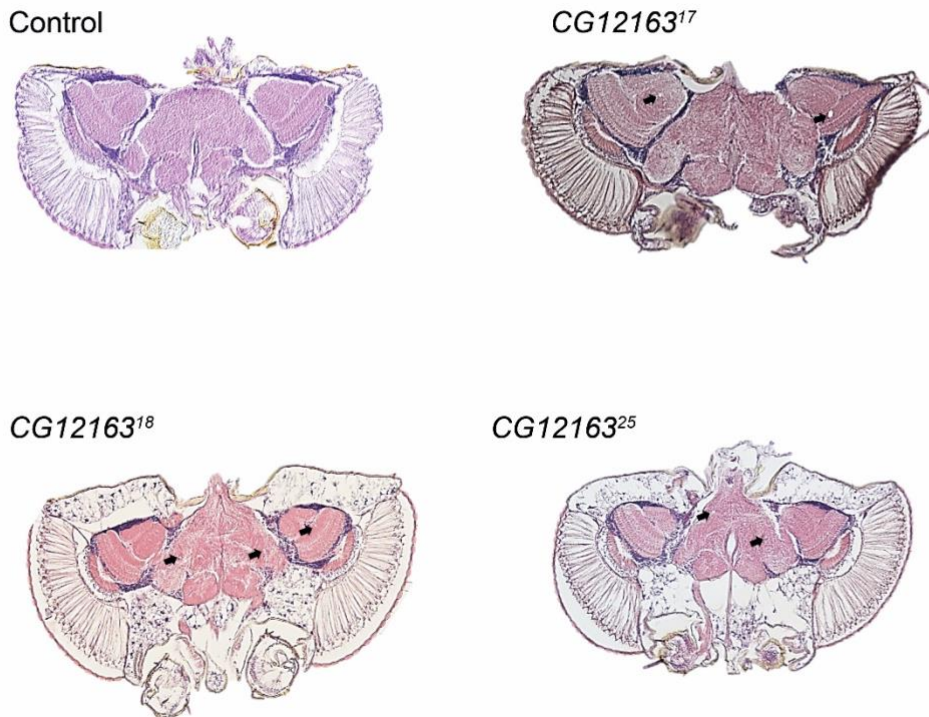
Figure 16: Adult brains of *CG12163* mutants display slightly increased brain vacuoles. Black arrows indicate position of vacuoles.

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).
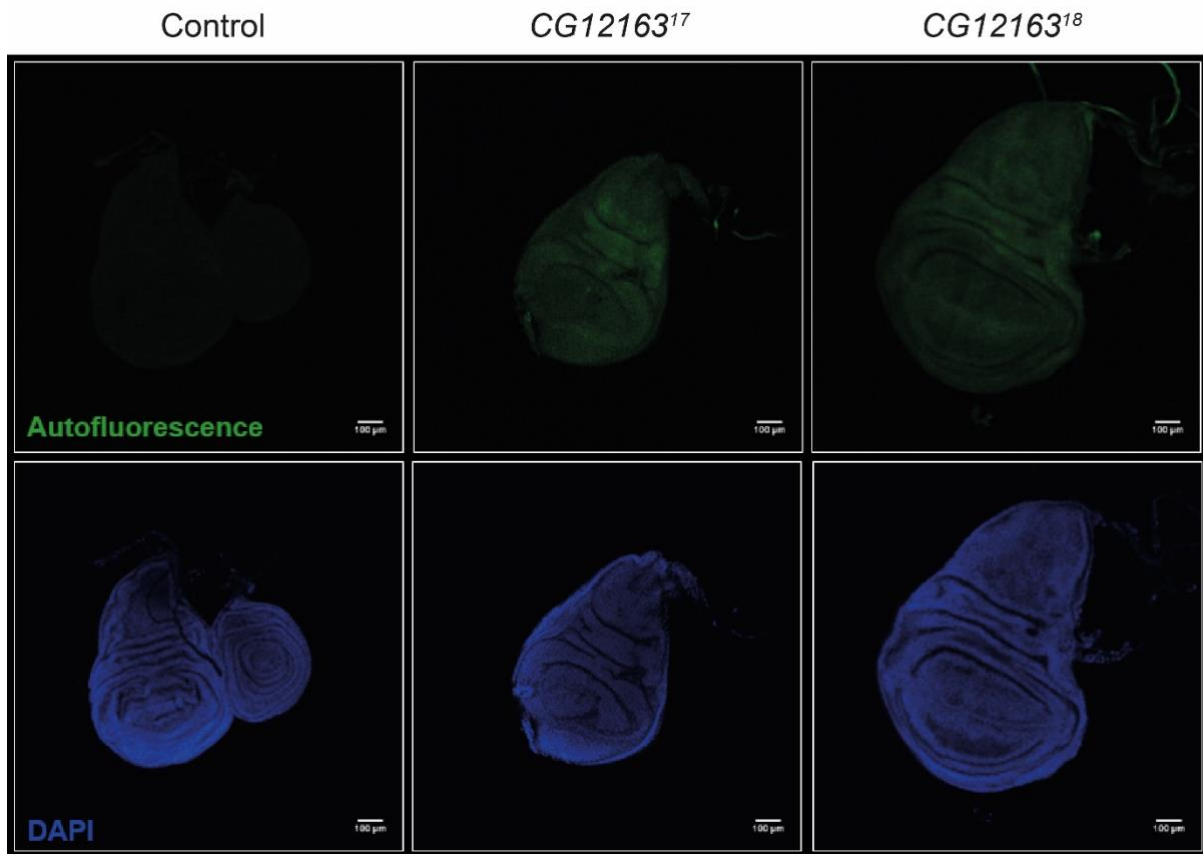
Figure 17: *CG12163* larval wing discs display autofluorescence.

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.
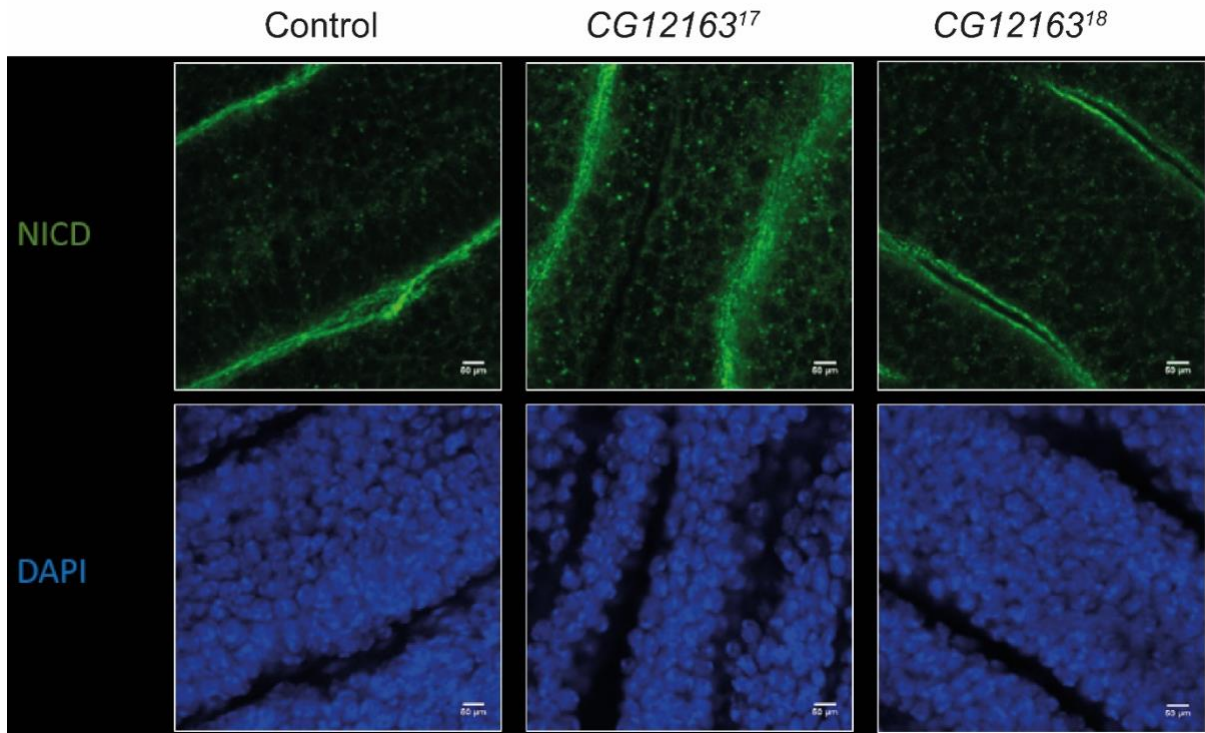
Figure 18: *CG12163* mutant wing discs do not display changes in Notch cleavage, based on levels of Notch intracellular domain (NICD)

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.
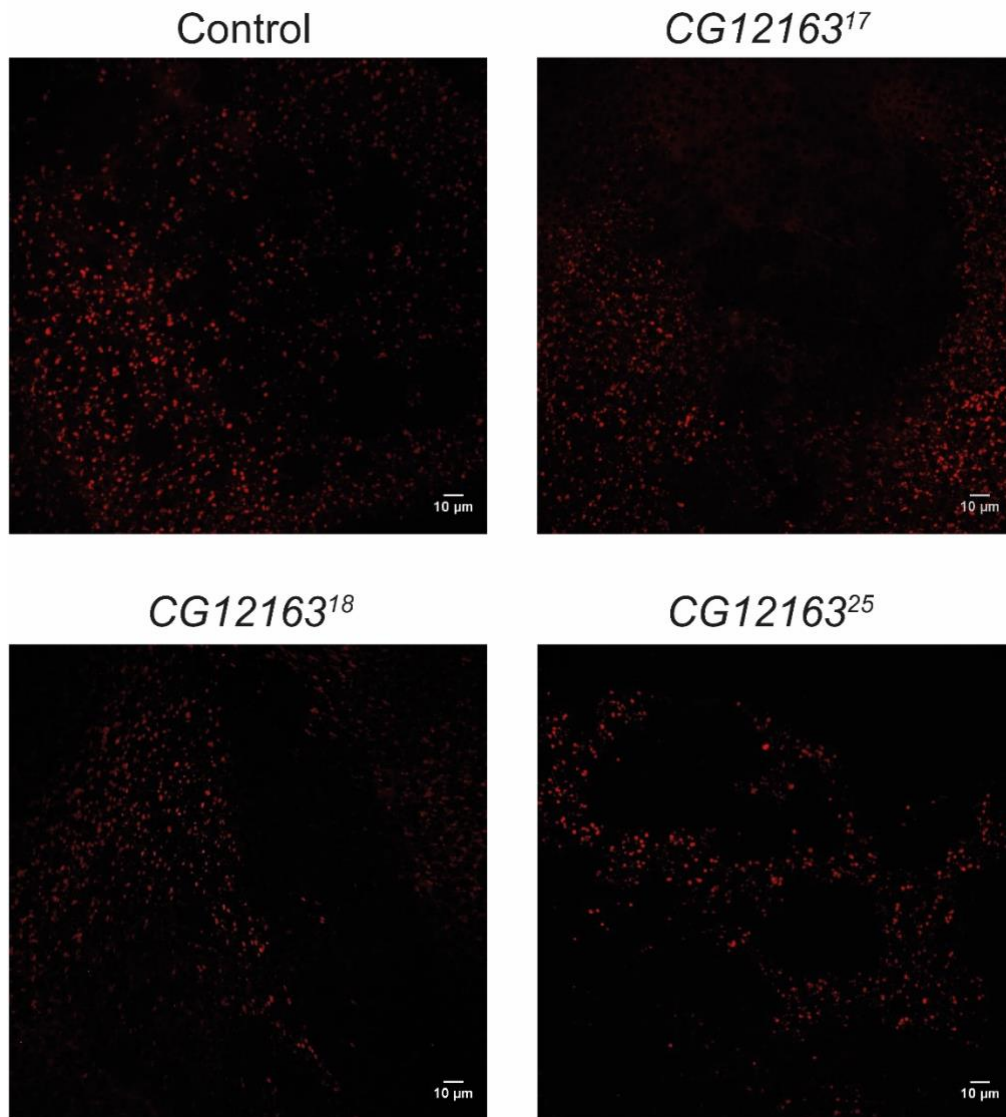
Figure 19*: CG12163 mutant larval wing discs do not display gross defects in lysosomal function, measured using the dqBSA assay.*

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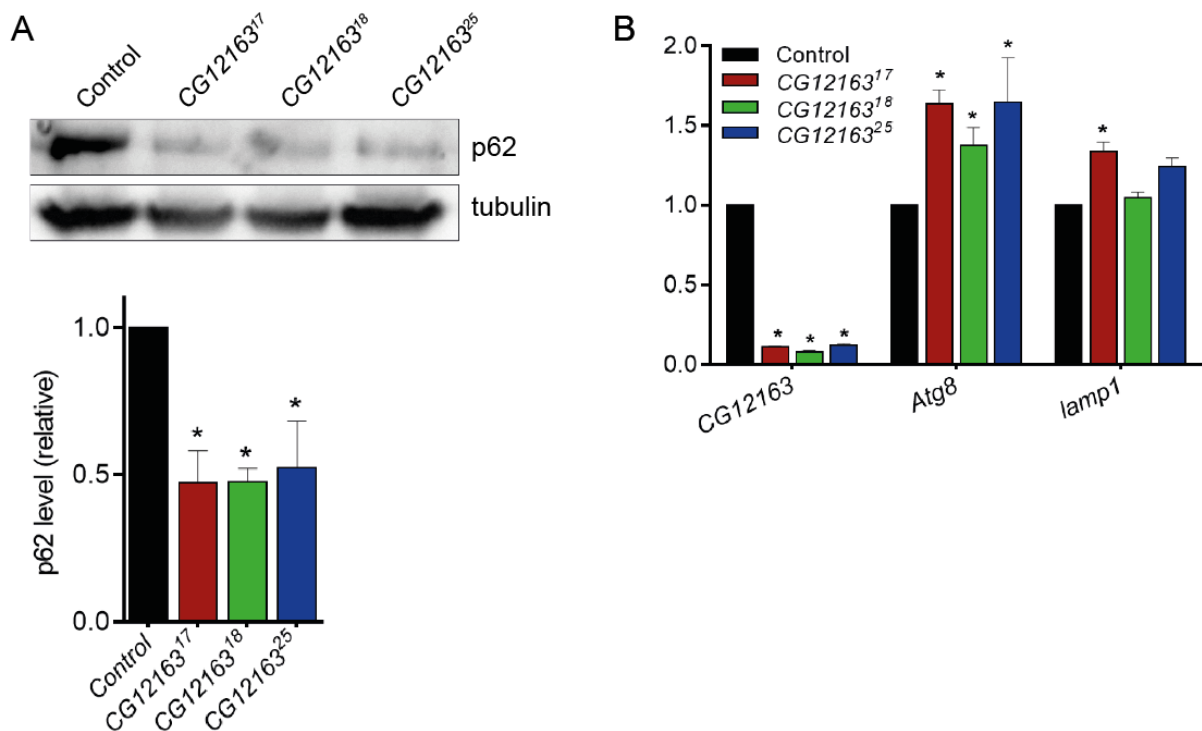
Figure 20: *CG12163* **mutant brains display alteration of autophagy**. (**A**) p62 protein
levels are reduced in 30-day old adult mutant heads. (**B**) *CG12163* mutant adult heads all
display transcriptional upregulation of *Atg8*, but not *Lamp1*.

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-Gaitan 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 (Dobbelaere 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 autophagic 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)mβ*. 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.,

2006). Consistent with the possibility that type B Kufs disease involve alteration of autophagy, we found changes in p62 expression in *CG12163* mutants. Rather than increase, we found reduction compared to control, suggesting that autophagy is somehow accelerated in *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). *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 *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 Atg8 is increased in *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 *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.

9 REFERENCES

Abrami, L., Brandi, L., Moayeri, M., Brown, M.J., Krantz, B.A., Leppla, S.H., and VanderGoot, F.G. (2013). Hijacking Multivesicular Bodies Enables Long-Term and Exosome-Mediated Long-Distance Action of Anthrax Toxin. Cell Rep. *5*, 986–996.

Adamowicz, M., Vermezovic, J., and d'Adda di Fagagna, F. (2016). NOTCH1 Inhibits Activation of ATM by Impairing the Formation of an ATM-FOXO3a-KAT5/Tip60 Complex. Cell Rep. *16*, 2068–2076.

Adell, M.A.Y., Vogel, G.F., Pakdel, M., Muller, M., Lindner, H., Hess, M.W., and Teis, D. (2014). Coordinated binding of Vps4 to ESCRT-III drives membrane neck constriction during MVB vesicle formation. J. Cell Biol. *205*, 33–49.

Agaisse, H., Burrack, L.S., Philips, J.A., Rubin, E.J., Perrimon, N., and Higgins, D.E. (2005). Genome-wide RNAi screen for host factors required for intracellular bacterial infection. Science *309*, 1248–1251.

Ai, E., Poole, D.S., and Skop, A.R. (2009). RACK-1 Directs Dynactin-dependent RAB-11 Endosomal Recycling during Mitosis in Caenorhabditis elegans. Mol. Biol. Cell *20*, 1629–1638.

Alenquer, M., and Amorim, M. (2015). Exosome Biogenesis, Regulation, and Function in Viral Infection. Viruses *7*, 5066–5083.

Alfred, V., and Vaccari, T. (2016). When membranes need an ESCRT: endosomal sorting and membrane remodelling in health and disease. Swiss Med. Wkly. *146*, w14347.

Alfred, V., and Vaccari, T. (2017). Mechanisms of non-canonical signaling in health and disease : Diversity to take therapy up a Notch ? In Molecular Mechanisms of Notch Signaling, T. Borggrefe, and B. Giaimo, eds. (Springer Nature (Accepted)), p.

Alvarez-Erviti, L., Seow, Y., Schapira, A.H., Gardiner, C., Sargent, I.L., Wood, M.J.A., and Cooper, J.M. (2011). Lysosomal dysfunction increases exosome-mediated alpha-synuclein release and transmission. Neurobiol. Dis. *42*, 360–367.

Archambault, V., Zhao, X., White-Cooper, H., Carpenter, A.T.C., and Glover, D.M. (2007). Mutations in Drosophila Greatwall/Scant Reveal Its Roles in Mitosis and Meiosis and Interdependence with Polo Kinase. PLoS Genet. *3*, e200.

Baba, M., Nakajo, S., Tu, P.H., Tomita, T., Nakaya, K., Lee, V.M., Trojanowski, J.Q., and Iwatsubo, T. (1998). Aggregation of alpha-synuclein in Lewy bodies of sporadic Parkinson's disease and dementia with Lewy bodies. Am. J. Pathol. *152*, 879–884.

Babst, M., Odorizzi, G., Estepa, E.J., and Emr, S.D. (2000). Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late

endosomal trafficking. Traffic *1*, 248–258.

Bache, K.G., Brech, A., Mehlum, A., and Stenmark, H. (2003). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. J. Cell Biol. *162*, 435–442.

Bache, K.G., Slagsvold, T., Cabezas, A., Rosendal, K.R., Raiborg, C., and Stenmark, H. (2004). The growth-regulatory protein HCRP1/hVps37A is a subunit of mammalian ESCRT-I and mediates receptor down-regulation. Mol. Biol. Cell *15*, 4337–4346.

Bache, K.G., Stuffers, S., Malerød, L., Slagsvold, T., Raiborg, C., Lechardeur, D., Wälchli, S., Lukacs, G.L., Brech, A., and Stenmark, H. (2006). The ESCRT-III subunit hVps24 is required for degradation but not silencing of the epidermal growth factor receptor. Mol. Biol. Cell *17*, 2513–2523.

Bader, R., Sarraf-Zadeh, L., Peters, M., Moderau, N., Stocker, H., Kohler, K., Pankratz, M.J., and Hafen, E. (2013). The IGFBP7 homolog Imp-L2 promotes insulin signaling in distinct neurons of the Drosophila brain. J. Cell Sci. *126*, 2571–2576.

Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E., et al. (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat. Cell Biol. *14*, 677–685.

Bakhoum, S.F., Kabeche, L., Compton, D.A., Powell, S.N., and Bastians, H. (2017). Mitotic DNA Damage Response: At the Crossroads of Structural and Numerical Cancer Chromosome Instabilities. Trends in Cancer *3*, 225–234.

Baldys, A., and Raymond, J.R. (2009). Critical role of ESCRT machinery in EGFR recycling. Biochemistry *48*, 9321–9323.

Bankaitis, V.A., Johnson, L.M., and Emr, S.D. (1986). Isolation of yeast mutants defective in protein targeting to the vacuole. Proc. Natl. Acad. Sci. U. S. A. *83*, 9075–9079.

Barlan, K., Lu, W., and Gelfand, V.I. (2013). The Microtubule-Binding Protein Ensconsin Is an Essential Cofactor of Kinesin-1. Curr. Biol. *23*, 317–322.

Bartlett, B.J., Isakson, P., Lewerenz, J., Sanchez, H., Kotzebue, R.W., Cumming, R.C., Harris, G.L., Nezis, I.P., Schubert, D.R., Simonsen, A., et al. (2011). p62, Ref(2)P and ubiquitinated proteins are conserved markers of neuronal aging, aggregate formation and progressive autophagic defects. Autophagy *7*, 572–583.

Basu, S., Thorat, R., and Dalal, S.N. (2015). MMP7 is required to mediate cell invasion and tumor formation upon plakophilin3 loss. PLoS One *10*, e0123979.

Bertrand, N., Castro, D.S., and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. Nat. Rev. Neurosci. *3*, 517–530.

Berx, G., and Van Roy, F. (2001). The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. Breast Cancer Res. *3*, 289–293.

Bièche, I., Laurendeau, I., Tozlu, S., Olivi, M., Vidaud, D., Lidereau, R., and Vidaud, M. (1999). Quantitation of MYC gene expression in sporadic breast tumors with a real-time reverse transcription-PCR assay. Cancer Res. *59*, 2759–2765.

Bishop, N., Horman, A., and Woodman, P. (2002). Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates. J. Cell Biol. *157*, 91–101.

Blanc, C., Charette, S.J., Mattei, S., Aubry, L., Smith, E.W., Cosson, P., and Letourneur, F. (2009). Dictyostelium Tom1 participates to an ancestral ESCRT-0 complex. Traffic *10*, 161–171.

Bleck, M., Itano, M.S., Johnson, D.S., Thomas, V.K., North, A.J., Bieniasz, P.D., and Simon, S.M. (2014). Temporal and spatial organization of ESCRT protein recruitment during HIV-1 budding. Proc. Natl. Acad. Sci. U. S. A. *111*, 12211–12216.

Bouché, V., Espinosa, A.P., Leone, L., Sardiello, M., Ballabio, A., and Botas, J. (2016). *Drosophila* Mitf regulates the V-ATPase and the lysosomal-autophagic pathway. Autophagy *12*, 484–498.

Braak, H., Tredici, K. Del, Rüb, U., de Vos, R.A.., Jansen Steur, E.N.., and Braak, E. (2003). Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol. Aging *24*, 197–211.

Bray, S.J. (2016). Notch signalling in context. Nat. Rev. Mol. Cell Biol. *17*, 722–735.

Broniarczyk, J., Olejnik-Schmidt, A.K., Luczak, M.W., Schmidt, M.T., Dabrowski, M., Józefiak, A., Kedzia, W., Kwasniewska, A., and Gozdzicka-Józefiak, A. (2010). Analysis of expression and structure of the TSG101 gene in cervical cancer cells. Int. J. Mol. Med. *25*, 777–783.

Broniarczyk, J., Bergant, M., Goździcka-Józefiak, A., and Banks, L. (2014). Human papillomavirus infection requires the TSG101 component of the ESCRT machinery. Virology *460–461*, 83–90.

Bryant, N.J., and Stevens, T.H. (1998). Vacuole biogenesis in Saccharomyces cerevisiae: protein transport pathways to the yeast vacuole. Microbiol. Mol. Biol. Rev. *62*, 230–247.

Bryant, P.J., and Schubiger, G. (1971). Giant and duplicated imaginal discs in a new lethal mutant of Drosophila melanogaster. Dev. Biol. *24*, 233–263.

Burgdorf, S., Leister, P., and Scheidtmann, K.H. (2004). TSG101 interacts with apoptosis-antagonizing transcription factor and enhances androgen receptor-mediated transcription by promoting its monoubiquitination. J. Biol. Chem. *279*, 17524–17534.

Burke, R., Nellen, D., Bellotto, M., Hafen, E., Senti, K.-A., Dickson, B.J., and Basler, K. (1999). Dispatched, a Novel Sterol-Sensing Domain Protein Dedicated to the Release of Cholesterol-Modified Hedgehog from Signaling Cells. Cell *99*, 803–815.

Busseau, I., Diederich, R.J., Xu, T., and Artavanis-Tsakonas, S. (1994). A member of the Notch group of interacting loci, deltex encodes a cytoplasmic basic protein. Genetics *136*, 585–596.

Capalbo, L., D'Avino, P.P., Archambault, V., and Glover, D.M. (2011). Rab5 GTPase controls chromosome alignment through Lamin disassembly and relocation of the NuMA-like protein Mud to the poles during mitosis. Proc. Natl. Acad. Sci. *108*, 17343–17348.

Carlton, J.G., and Martin-Serrano, J. (2007). Parallels between cytokinesis and retroviral budding: a role for the ESCRT machinery. Science *316*, 1908–1912.

Cavalier-Smith, T. (2002). The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. Int. J. Syst. Evol. Microbiol. *52*, 297–354.

Chanut-Delalande, H., Jung, A.C., Baer, M.M., Lin, L., Payre, F., and Affolter, M. (2010). The Hrs/Stam complex acts as a positive and negative regulator of RTK signaling during Drosophila development. PLoS One *5*, e10245.

Chastagner, P., Israël, A., and Brou, C. (2006). Itch/AIP4 mediates Deltex degradation through the formation of K29-linked polyubiquitin chains. EMBO Rep. *7*, 1147–1153.

Chaves, K.C.B., Turaça, L.T., Pesquero, J.B., Mennecier, G., Dagli, M.L.Z., Chammas, R., Schor, N., and Bellini, M.H. (2012). Fibronectin expression is decreased in metastatic renal cell carcinoma following endostatin gene therapy. Biomed. Pharmacother. *66*, 464–468.

Chen, R.-H. (2002). BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. J. Cell Biol. *158*, 487–496.

Chen, C.-T., Hehnly, H., and Doxsey, S.J. (2012). Orchestrating vesicle transport, ESCRTs and kinase surveillance during abscission. Nat. Rev. Mol. Cell Biol. *13*, 483–488.

Chen, F., Deng, J., Liu, X., Li, W., and Zheng, J. (2015). HCRP-1 regulates cell migration and invasion via EGFR-ERK mediated up-regulation of MMP-2 with prognostic significance in human renal cell carcinoma. Sci. Rep. *5*, 13470.

Chen, J., Futami, K., Petillo, D., Peng, J., Wang, P., Knol, J., Li, Y., Khoo, S.-K.,

Huang, D., Qian, C.-N., et al. (2008). Deficiency of FLCN in Mouse Kidney Led to Development of Polycystic Kidneys and Renal Neoplasia. PLoS One *3*, e3581.

Chen, V.Y., Posada, M.M., Blazer, L.L., Zhao, T., and Rosania, G.R. (2006). The role of the VPS4A-exosome pathway in the intrinsic egress route of a DNA-binding anticancer drug. Pharm. Res. *23*, 1687–1695.

Cheng, S., Tada, M., Hida, Y., Asano, T., Kuramae, T., Takemoto, N., Hamada, J.-I., Miyamoto, M., Hirano, S., Kondo, S., et al. (2008). High MMP-1 mRNA Expression is a Risk Factor for Disease-Free and Overall Survivals in Patients with Invasive Breast Carcinoma. J. Surg. Res. *146*, 104–109.

Childress, J.L., Acar, M., Tao, C., and Halder, G. (2006). Lethal Giant Discs, a Novel C2-Domain Protein, Restricts Notch Activation during Endocytosis. Curr. Biol. *16*, 2228–2233.

Choudhuri, K., Llodrá, J., Roth, E.W., Tsai, J., Gordo, S., Wucherpfennig, K.W., Kam, L.C., Stokes, D.L., and Dustin, M.L. (2014). Polarized release of T-cell-receptor-enriched microvesicles at the immunological synapse. Nature *507*, 118–123.

Clay, M.R., Varma, S., and West, R.B. (2013). MAST2 and NOTCH1 translocations in breast carcinoma and associated pre-invasive lesions. Hum. Pathol. *44*, 2837–2844.

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L.F., Théry, C., and Raposo, G. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J. Cell Sci. *126*, 5553–5565.

Constam, D.B. (2009). Intracellular trafficking and signaling in development. F1000 Biol. Rep. *1*, 59.

Corless, L., Crump, C.M., Griffin, S.D.C., and Harris, M. (2010). Vps4 and the ESCRT-III complex are required for the release of infectious hepatitis C virus particles. J. Gen. Virol. *91*, 362–372.

Cornell, M., Evans, D.A., Mann, R., Fostier, M., Flasza, M., Monthatong, M., Artavanis-Tsakonas, S., and Baron, M. (1999). The Drosophila melanogaster Suppressor of deltex gene, a regulator of the Notch receptor signaling pathway, is an E3 class ubiquitin ligase. Genetics *152*, 567–576.

Cornet, M., Bidard, F., Schwarz, P., Da Costa, G., Blanchin-Roland, S., Dromer, F., and Gaillardin, C. (2005). Deletions of endocytic components VPS28 and VPS32 affect growth at alkaline pH and virulence through both RIM101-dependent and RIM101-independent pathways in Candida albicans. Infect. Immun. *73*, 7977–7987.

Cox, L.E., Ferraiuolo, L., Goodall, E.F., Heath, P.R., Higginbottom, A., Mortiboys, H., Hollinger, H.C., Hartley, J.A., Brockington, A., Burness, C.E., et al. (2010). Mutations in CHMP2B in lower motor neuron predominant amyotrophic lateral sclerosis (ALS). PLoS One *5*, e9872.

Dacks, J.B., and Field, M.C. (2007). Evolution of the eukaryotic membrane-trafficking system: origin, tempo and mode. J. Cell Sci. *120*, 2977–2985.

Dalton, H.E., Denton, D., Foot, N.J., Ho, K., Mills, K., Brou, C., and Kumar, S. (2011). Drosophila Ndfip is a novel regulator of Notch signaling. Cell Death Differ. *18*, 1150–1160.

Decock, J., Hendrickx, W., Drijkoningen, M., Wildiers, H., Neven, P., Smeets, A., and Paridaens, R. (2007). Matrix metalloproteinase expression patterns in luminal A type breast carcinomas. Dis. Markers *23*, 189–196.

Delgehyr, N., Wieland, U., Rangone, H., Pinson, X., Mao, G., Dzhindzhev, N.S., McLean, D., Riparbelli, M.G., Llamazares, S., Callaini, G., et al. (2012). Drosophila Mgr, a Prefoldin subunit cooperating with von Hippel Lindau to regulate tubulin stability. Proc. Natl. Acad. Sci. U. S. A. *109*, 5729–5734.

Deshar, R., Cho, E.-B., Yoon, S.K., and Yoon, J.-B. (2016). CC2D1A and CC2D1B regulate degradation and signaling of EGFR and TLR4. Biochem. Biophys. Res. Commun. *480*, 280–287.

Dieckmann, A.K., Babin, V., Harari, Y., Eils, R., König, R., Luke, B., and Kupiec, M. (2016). Role of the ESCRT Complexes in Telomere Biology. MBio *7*, e01793-16.

Diederich, R.J., Matsuno, K., Hing, H., and Artavanis-Tsakonas, S. (1994). Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. Development *120*, 473–481.

Ding, Y., Yao, C., Lince-Faria, M., Rath, U., Cai, W., Maiato, H., Girton, J., Johansen, K.M., and Johansen, J. (2009). Chromator is required for proper microtubule spindle formation and mitosis in Drosophila. Dev. Biol. *334*, 253–263.

Djiane, A., Shimizu, H., Wilkin, M., Mazleyrat, S., Jennings, M.D., Avis, J., Bray, S., and Baron, M. (2011). Su(dx) E3 ubiquitin ligase-dependent and -independent functions of polychaetoid, the Drosophila ZO-1 homologue. J. Cell Biol. *192*, 189–200.

Dobbelaere, J., Josué, F., Suijkerbuijk, S., Baum, B., Tapon, N., and Raff, J. (2008). A Genome-Wide RNAi Screen to Dissect Centriole Duplication and Centrosome Maturation in Drosophila. PLoS Biol. *6*, e224.

Dominguez, M., Ferres-Marco, D., Gutierrez-Aviño, F.J., Speicher, S.A., and

Beneyto, M. (2004). Growth and specification of the eye are controlled independently by Eyegone and Eyeless in Drosophila melanogaster. Nat. Genet. *36*, 31–39.

Doyotte, A., Russell, M.R.G., Hopkins, C.R., and Woodman, P.G. (2005). Depletion of TSG101 forms a mammalian "Class E" compartment: a multicisternal early endosome with multiple sorting defects. J. Cell Sci. *118*, 3003–3017.

Drusenheimer, N., Migdal, B., Jäckel, S., Tveriakhina, L., Scheider, K., Schulz, K., Gröper, J., Köhrer, K., and Klein, T. (2015). The Mammalian Orthologs of Drosophila Lgd, CC2D1A and CC2D1B, Function in the Endocytic Pathway, but Their Individual Loss of Function Does Not Affect Notch Signalling. PLoS Genet. *11*, e1005749.

Dukes, J.D., Fish, L., Richardson, J.D., Blaikley, E., Burns, S., Caunt, C.J., Chalmers, A.D., and Whitley, P. (2011). Functional ESCRT machinery is required for constitutive recycling of claudin-1 and maintenance of polarity in vertebrate epithelial cells. Mol. Biol. Cell *22*, 3192–3205.

Edgar, R.C. (2004a). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. *32*, 1792–1797.

Edgar, R.C. (2004b). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics *5*, 113.

Edgar, B.A., and O'Farrell, P.H. (1989). Genetic control of cell division patterns in the Drosophila embryo. Cell *57*, 177–187.

Edgar, B.A., and O'Farrell, P.H. (1990). The three postblastoderm cell cycles of Drosophila embryogenesis are regulated in G2 by string. Cell *62*, 469–480.

Edgar, J.R., Willén, K., Gouras, G.K., and Futter, C.E. (2015). ESCRTs regulate amyloid precursor protein sorting in multivesicular bodies and intracellular amyloid-β accumulation. J. Cell Sci. *128*, 2520–2528.

Effantin, G., Dordor, A., Sandrin, V., Martinelli, N., Sundquist, W.I., Schoehn, G., and Weissenhorn, W. (2013). ESCRT-III CHMP2A and CHMP3 form variable helical polymers in vitro and act synergistically during HIV-1 budding. Cell. Microbiol. *15*, 213–226.

Emre, D., Terracol, R., Poncet, A., Rahmani, Z., and Karess, R.E. (2011). A mitotic role for Mad1 beyond the spindle checkpoint. J. Cell Sci. *124*, 1664–1671.

Van Engelenburg, S.B., Shtengel, G., Sengupta, P., Waki, K., Jarnik, M., Ablan, S.D., Freed, E.O., Hess, H.F., and Lippincott-Schwartz, J. (2014). Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits. Science *343*, 653–656.

Evron, E., Umbricht, C.B., Korz, D., Raman, V., Loeb, D.M., Niranjan, B., Buluwela, L., Weitzman, S.A., Marks, J., and Sukumar, S. (2001). Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. Cancer Res. *61*, 2782–2787.

Di Fabio, R., Moro, F., Pestillo, L., Meschini, M.C., Pezzini, F., Doccini, S., Casali, C., Pierelli, F., Simonati, A., and Santorelli, F.M. (2014). Pseudo-dominant inheritance of a novel CTSF mutation associated with type B Kufs disease. Neurology *83*, 1769–1770.

Di Fabio, R., Colonnese, C., Santorelli, F.M., Pestillo, L., and Pierelli, F. (2015). Brain imaging in Kufs disease type B: case reports. BMC Neurol. *15*, 102.

Feng, Y., and Klionsky, D.J. (2017). Autophagy regulates DNA repair through SQSTM1/p62. Autophagy *13*, 995–996.

Feng, G.H., Lih, C.J., and Cohen, S.N. (2000). TSG101 protein steady-state level is regulated posttranslationally by an evolutionarily conserved COOH-terminal sequence. Cancer Res. *60*, 1736–1741.

Ferres-Marco, D., Gutierrez-Garcia, I., Vallejo, D.M., Bolivar, J., Gutierrez-Aviño, F.J., and Dominguez, M. (2006). Epigenetic silencers and Notch collaborate to promote malignant tumours by Rb silencing. Nature *439*, 430–436.

Field, M.C., and Dacks, J.B. (2009). First and last ancestors: reconstructing evolution of the endomembrane system with ESCRTs, vesicle coat proteins, and nuclear pore complexes. Curr. Opin. Cell Biol. *21*, 4–13.

Filimonenko, M., Stuffers, S., Raiborg, C., Yamamoto, A., Malerød, L., Fisher, E.M.C., Isaacs, A., Brech, A., Stenmark, H., and Simonsen, A. (2007). Functional multivesicular bodies are required for autophagic clearance of protein aggregates associated with neurodegenerative disease. J. Cell Biol. *179*, 485–500.

Fischer, H., Chen, J., Skoog, L., and Lindblom, A. (2002). Cyclin D2 expression in familial and sporadic breast cancer. Oncol Rep *9*, 1157–1161.

Foda, H.D., and Zucker, S. (2001). Matrix metalloproteinases in cancer invasion, metastasis and angiogenesis. Drug Discov. Today *6*, 478–482.

Fostier, M., Evans, D.A., Artavanis-Tsakonas, S., and Baron, M. (1998). Genetic characterization of the Drosophila melanogaster Suppressor of deltex gene: A regulator of notch signaling. Genetics *150*, 1477–1485.

Frost, A., Elgort, M.G., Brandman, O., Ives, C., Collins, S.R., Miller-Vedam, L., Weibezahn, J., Hein, M.Y., Poser, I., Mann, M., et al. (2012). Functional repurposing revealed by comparing S. pombe and S. cerevisiae genetic interactions. Cell *149*, 1339–1352.

Funakoshi-Tago, M., Kamada, N., Shimizu, T., Hashiguchi, Y., Tago, K., Sonoda, Y., and Kasahara, T. (2009). TRAF6 negatively regulates TNFα-induced NF-κB activation. Cytokine *45*, 72–79.

Galewsky, S., and Schulz, R.A. (1992). Drop out: a third chromosome maternal-effect locus required for formation of the Drosophila cellular blastoderm. Mol. Reprod. Dev. *32*, 331–338.

Gallagher, C.M., and Knoblich, J.A. (2006). The Conserved C2 Domain Protein Lethal (2) Giant Discs Regulates Protein Trafficking in Drosophila. Dev. Cell *11*, 641–653.

Gallaud, E., Caous, R., Pascal, A., Bazile, F., Gagné, J.-P., Huet, S., Poirier, G.G., Chrétien, D., Richard-Parpaillon, L., and Giet, R. (2014). Ensconsin/Map7 promotes microtubule growth and centrosome separation in *Drosophila* neural stem cells. J. Cell Biol. *204*, 1111–1121.

Ganem, N.J., and Pellman, D. (2012). Linking abnormal mitosis to the acquisition of DNA damage. J. Cell Biol. *199*, 871–881.

Garrison, A.R., Radoshitzky, S.R., Kota, K.P., Pegoraro, G., Ruthel, G., Kuhn, J.H., Altamura, L.A., Kwilas, S.A., Bavari, S., Haucke, V., et al. (2013). Crimean-Congo hemorrhagic fever virus utilizes a clathrin- and early endosome-dependent entry pathway. Virology *444*, 45–54.

Garrus, J.E., von Schwedler, U.K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, D.A., Stray, K.M., Côté, M., Rich, R.L., et al. (2001). Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell *107*, 55–65.

Gaur, K., Li, J., Wang, D., Dutta, P., Yan, S.-J., Tsurumi, A., Land, H., Wu, G., and Li, W.X. (2013). The Birt-Hogg-Dube tumor suppressor Folliculin negatively regulates ribosomal RNA synthesis. Hum. Mol. Genet. *22*, 284–299.

Ghabrial, A., Ray, R.P., and Schüpbach, T. (1998). okra and spindle-B encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in Drosophila oogenesis. Genes Dev. *12*, 2711–2723.

Ghoujal, B., Milev, M.P., Ajamian, L., Abel, K., and Mouland, A.J. (2012). ESCRT-II's involvement in HIV-1 genomic RNA trafficking and assembly. Biol. Cell *104*, 706–721.

Gibbings, D.J., Ciaudo, C., Erhardt, M., and Voinnet, O. (2009). Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. Nat. Cell Biol. *11*, 1143–1149.

Gilbert, M.M., Robinson, B.S., and Moberg, K.H. (2009). Functional interactions between the erupted/tsg101 growth suppressor gene and the DaPKC and rbf1 genes in

Drosophila imaginal disc tumors. PLoS One *4*, e7039.

Gilmore, T.D. (2006). Introduction to NF-κB: players, pathways, perspectives. Oncogene *25*, 6680–6684.

Goliand, I., Nachmias, D., Gershony, O., and Elia, N. (2014). Inhibition of ESCRT-II-CHMP6 interactions impedes cytokinetic abscission and leads to cell death. Mol. Biol. Cell *25*, 3740–3748.

Gonzalez, C., Sunkel, C.E., and Glover, D.M. (1998). Interactions between mgr, asp, and polo: asp function modulated by polo and needed to maintain the poles of monopolar and bipolar spindles. Chromosoma *107*, 452–460.

González, C., Casal, J., and Ripoll, P. (1988). Functional monopolar spindles caused by mutation in mgr, a cell division gene of Drosophila melanogaster. J. Cell Sci. *89 (Pt 1)*, 39–47.

Gonzalez-Gaitan, M., and González-Gaitán, M. (2003). Signal dispersal and transduction through the endocytic pathway. Nat Rev Mol Cell Biol *4*, 213–224.

Haglund, K., and Dikic, I. (2012). The role of ubiquitylation in receptor endocytosis and endosomal sorting. J. Cell Sci. *125*, 265–275.

Hain, D., Langlands, A., Sonnenberg, H.C., Bailey, C., Bullock, S.L., and Müller, H.-A.J. (2014). The Drosophila MAST kinase Drop out is required to initiate membrane compartmentalisation during cellularisation and regulates dynein-based transport. Development *141*, 2119–2130.

Handschuh, K., Feenstra, J., Koss, M., Ferretti, E., Risolino, M., Zewdu, R., Sahai, M.A., Bénazet, J.-D., Peng, X.P., Depew, M.J., et al. (2014). ESCRT-II/Vps25 constrains digit number by endosome-mediated selective modulation of FGF-SHH signaling. Cell Rep. *9*, 674–687.

Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., et al. (2006). Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature *441*, 885–889.

Hasegawa, T., Konno, M., Baba, T., Sugeno, N., Kikuchi, A., Kobayashi, M., Miura, E., Tanaka, N., Tamai, K., Furukawa, K., et al. (2011). The AAA-ATPase VPS4 regulates extracellular secretion and lysosomal targeting of α-synuclein. PLoS One *6*, e29460.

Hasumi, H., Baba, M., Hong, S.-B., Hasumi, Y., Huang, Y., Yao, M., Valera, V.A., Linehan, W.M., and Schmidt, L.S. (2008). Identification and characterization of a novel folliculin-interacting protein FNIP2. Gene *415*, 60–67.

Hasumi, H., Baba, M., Hasumi, Y., Lang, M., Huang, Y., Oh, H.F., Matsuo, M., Merino, M.J., Yao, M., Ito, Y., et al. (2015). Folliculin-interacting proteins Fnip1 and Fnip2 play critical roles in kidney tumor suppression in cooperation with Flcn. Proc. Natl. Acad. Sci. U. S. A. *112*, E1624-31.

He, C. (1996). Molecular mechanism of transcriptional activation of human gelatinase B by proximal promoter. Cancer Lett. *106*, 185–191.

Heijink, A.M., Krajewska, M., and van Vugt, M.A.T.M. (2013). The DNA damage response during mitosis. Mutat. Res. Mol. Mech. Mutagen. *750*, 45–55.

Henne, W.M., Buchkovich, N.J., Zhao, Y., and Emr, S.D. (2012). The endosomal sorting complex ESCRT-II mediates the assembly and architecture of ESCRT-III helices. Cell *151*, 356–371.

Herz, H.-M., Chen, Z., Scherr, H., Lackey, M., Bolduc, C., and Bergmann, A. (2006). Vps25 Mosaics Display Non-Autonomous Cell Survival and Overgrowth, and Autonomous Apoptosis. Development *133*, 1871–1880.

Herz, H.-M., Woodfield, S.E., Chen, Z., Bolduc, C., and Bergmann, A. (2009). Common and distinct genetic properties of ESCRT-II components in Drosophila. PLoS One *4*, e4165.

Herz, H.M., Madden, L.D., Chen, Z., Bolduc, C., Buff, E., Gupta, R., Davuluri, R., Shilatifard, A., Hariharan, I.K., and Bergmann, A. (2010). The H3K27me3 Demethylase dUTX Is a Suppressor of Notch- and Rb-Dependent Tumors in Drosophila. Mol. Cell. Biol. *30*, 2485–2497.

Hierro, A., Sun, J., Rusnak, A.S., Kim, J., Prag, G., Emr, S.D., and Hurley, J.H. (2004). Structure of the ESCRT-II endosomal trafficking complex. Nature *431*, 221–225.

Holm, I.E., Englund, E., Mackenzie, I.R.A., Johannsen, P., and Isaacs, A.M. (2007). A reassessment of the neuropathology of frontotemporal dementia linked to chromosome 3. J. Neuropathol. Exp. Neurol. *66*, 884–891.

Holopainen, J.M., Saarikoski, J., Kinnunen, P.K., and Järvelä, I. (2001). Elevated lysosomal pH in neuronal ceroid lipofuscinoses (NCLs). Eur. J. Biochem. *268*, 5851–5856.

Hong, S., and Laimins, L.A. (2013). The JAK-STAT Transcriptional Regulator, STAT-5, Activates the ATM DNA Damage Pathway to Induce HPV 31 Genome Amplification upon Epithelial Differentiation. PLoS Pathog. *9*, e1003295.

Hori, K., Fostier, M., Ito, M., Fuwa, T.J., Go, M.J., Okano, H., Baron, M., and Matsuno, K. (2004). Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling. Development *131*, 5527–5537.

Hori, K., Sen, A., Kirchhausen, T., and Artavanis-Tsakonas, S. (2011). Synergy between the ESCRT-III complex and Deltex defines a ligand-independent Notch signal. J. Cell Biol. *195*, 1005–1015.

Hu, B., Jiang, D., Chen, Y., Wei, L., Zhang, S., Zhao, F., Ni, R., Lu, C., and Wan, C. (2015). High CHMP4B expression is associated with accelerated cell proliferation and resistance to doxorubicin in hepatocellular carcinoma. Tumor Biol. *36*, 2569–2581.

Huang, H.-R., Chen, Z.J., Kunes, S., Chang, G.-D., and Maniatis, T. (2010). Endocytic pathway is required for Drosophila Toll innate immune signaling. Proc. Natl. Acad. Sci. U. S. A. *107*, 8322–8327.

Hudon, V., Sabourin, S., Dydensborg, A.B., Kottis, V., Ghazi, A., Paquet, M., Crosby, K., Pomerleau, V., Uetani, N., and Pause, A. (2010). Renal tumour suppressor function of the Birt-Hogg-Dube syndrome gene product folliculin. J. Med. Genet. *47*, 182–189.

Hurley, J.H. (2010). The ESCRT complexes. Crit. Rev. Biochem. Mol. Biol. *45*, 463–487.

Im, Y.J., and Hurley, J.H. (2008). Integrated structural model and membrane targeting mechanism of the human ESCRT-II complex. Dev. Cell *14*, 902–913.

Irion, U., and St Johnston, D. (2007). bicoid RNA localization requires specific binding of an endosomal sorting complex. Nature *445*, 554–558.

Issman-Zecharya, N., and Schuldiner, O. (2014). The PI3K class III complex promotes axon pruning by downregulating a Ptc-derived signal via endosome-lysosomal degradation. Dev. Cell *31*, 461–473.

Jaekel, R., and Klein, T. (2006). The Drosophila Notch inhibitor and tumor suppressor gene lethal (2) giant discs encodes a conserved regulator of endosomal trafficking. Dev. Cell *11*, 655–669.

Jékely, G., and Rørth, P. (2003). Hrs mediates downregulation of multiple signalling receptors in Drosophila. EMBO Rep. *4*, 1163–1168.

Jimenez, A.J., Maiuri, P., Lafaurie-Janvore, J., Divoux, S., Piel, M., and Perez, F. (2014). ESCRT machinery is required for plasma membrane repair. Science *343*, 1247136.

Jin, Y., Mancuso, J.J., Uzawa, S., Cronembold, D., and Cande, W.Z. (2005). The fission yeast homolog of the human transcription factor EAP30 blocks meiotic spindle pole body amplification. Dev. Cell *9*, 63–73.

Jones, C.B., Ott, E.M., Keener, J.M., Curtiss, M., Sandrin, V., and Babst, M. (2012). Regulation of membrane protein degradation by starvation-response pathways. Traffic *13*,

468–482.

Jun, M.-H., Han, J.-H., Lee, Y.-K., Jang, D.-J., Kaang, B.-K., and Lee, J.-A. (2015). TMEM106B, a frontotemporal lobar dementia (FTLD) modifier, associates with FTD-3-linked CHMP2B, a complex of ESCRT-III. Mol. Brain *8*, 85.

Kadiu, I., Narayanasamy, P., Dash, P.K., Zhang, W., and Gendelman, H.E. (2012). Biochemical and biologic characterization of exosomes and microvesicles as facilitators of HIV-1 infection in macrophages. J. Immunol. *189*, 744–754.

Kamura, T., Burian, D., Khalili, H., Schmidt, S.L., Sato, S., Liu, W.J., Conrad, M.N., Conaway, R.C., Conaway, J.W., and Shilatifard, A. (2001). Cloning and characterization of ELL-associated proteins EAP45 and EAP20. a role for yeast EAP-like proteins in regulation of gene expression by glucose. J. Biol. Chem. *276*, 16528–16533.

Karin, M. (2006). Nuclear factor-κB in cancer development and progression. Nature *441*, 431–436.

Katzmann, D.J., Babst, M., and Emr, S.D. (2001). Ubiquitin-Dependent Sorting into the Multivesicular Body Pathway Requires the Function of a Conserved Endosomal Protein Sorting Complex, ESCRT-I. Cell *106*, 145–155.

Kaur, J., and Debnath, J. (2015). Autophagy at the crossroads of catabolism and anabolism. Nat. Rev. Mol. Cell Biol. *16*, 461–472.

Kim, J.H., Park, S.-M., Kang, M.R., Oh, S.-Y., Lee, T.H., Muller, M.T., and Chung, I.K. (2005). Ubiquitin ligase MKRN1 modulates telomere length homeostasis through a proteolysis of hTERT. Genes Dev. *19*, 776–781.

Klein, T. (2003). The tumour suppressor gene l(2)giant discs is required to restrictthe activity of Notch to the dorsoventral boundaryduring Drosophila wing development. Dev. Biol. *255*, 313–333.

Kohrmann, A., Kammerer, U., Kapp, M., Dietl, J., and Anacker, J. (2009). Expression of matrix metalloproteinases (MMPs) in primary human breast cancer and breast cancer cell lines: New findings and review of the literature. BMC Cancer *9*, 188.

Koike, M., Nakanishi, H., Saftig, P., Ezaki, J., Isahara, K., Ohsawa, Y., Schulz-Schaeffer, W., Watanabe, T., Waguri, S., Kametaka, S., et al. (2000). Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscin in mouse CNS neurons. J. Neurosci. *20*, 6898–6906.

Komada, M., and Soriano, P. (1999). Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis. Genes Dev. *13*, 1475–1485.

Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., et al. (2006). Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature *441*, 880–884.

Kondo, S., and Ueda, R. (2013). Highly Improved Gene Targeting by Germline-Specific Cas9 Expression in *Drosophila*. Genetics *195*, 715–721.

Kousidou, O.C., Roussidis, A.E., Theocharis, A.D., and Karamanos, N.K. (2004). Expression of MMPs and TIMPs genes in human breast cancer epithelial cells depends on cell culture conditions and is associated with their invasive potential. Anticancer Res. *24*, 4025–4030.

Krause, S.U.E.A., Loupart, M., Vass, S., Schoenfelder, S., Harrison, S., and Heck, M.M.S. (2001). Loss of Cell Cycle Checkpoint Control in Drosophila Rfc4 Mutants. *21*, 5156–5168.

Krempler, A., Henry, M.D., Triplett, A.A., and Wagner, K.-U. (2002). Targeted deletion of the Tsg101 gene results in cell cycle arrest at G1/S and p53-independent cell death. J. Biol. Chem. *277*, 43216–43223.

Krenn, V., and Musacchio, A. (2015). The Aurora B Kinase in Chromosome Bi-Orientation and Spindle Checkpoint Signaling. Front. Oncol. *5*, 225.

Kullas, A.L., Li, M., and Davis, D.A. (2004). Snf7p, a component of the ESCRT-III protein complex, is an upstream member of the RIM101 pathway in Candida albicans. Eukaryot. Cell *3*, 1609–1618.

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. *33*, 1870–1874.

Kurashige, T., Takahashi, T., Yamazaki, Y., Hiji, M., Izumi, Y., Yamawaki, T., and Matsumoto, M. (2013). Localization of CHMP2B-immunoreactivity in the brainstem of Lewy body disease. Neuropathology *33*, 237–245.

Kuronen, M., Talvitie, M., Lehesjoki, A.-E., and Myllykangas, L. (2009). Genetic modifiers of degeneration in the cathepsin D deficient Drosophila model for neuronal ceroid lipofuscinosis. Neurobiol. Dis. *36*, 488–493.

Labbé, C., Goyette, P., Lefebvre, C., Stevens, C., Green, T., Tello-Ruiz, M.K., Cao, Z., Landry, A.L., Stempak, J., Annese, V., et al. (2008). MAST3: a novel IBD risk factor that modulates TLR4 signaling. Genes Immun. *9*, 602–612.

Labbé, C., Boucher, G., Foisy, S., Alikashani, A., Nkwimi, H., David, G., Beaudoin, M., Goyette, P., Charron, G., Xavier, R.J., et al. (2012). Genome-wide expression profiling implicates a MAST3-regulated gene set in colonic mucosal inflammation of ulcerative colitis

patients. Inflamm. Bowel Dis. *18*, 1072–1080.

Lai, M.W., Huang, S.F., Lin, S.M., Chen, T.C., Lin, C.Y., Yeh, C.N., Yeh, T. Sen, Chen, M.F., and Yeh, C.T. (2009). Expression of the HCRP1 mRNA in HCC as an independent predictor of disease-free survival after surgical resection. Hepatol. Res. *39*, 164–176.

Lamb, C.A., Yoshimori, T., and Tooze, S.A. (2013). The autophagosome: origins unknown, biogenesis complex. Nat. Rev. Mol. Cell Biol. *14*, 759–774.

Lamouille, S., Xu, J., and Derynck, R. (2014). Molecular mechanisms of epithelial-mesenchymal transition. Nat. Rev. Mol. Cell Biol. *15*, 178–196.

Lanzetti, L. (2012). A novel function of Rab5 in mitosis. Small GTPases *3*, 168–172.

Lee, J.-A. (2012). Neuronal autophagy: a housekeeper or a fighter in neuronal cell survival? Exp. Neurobiol. *21*, 1–8.

Lee, Y.S., and Dutta, A. (2009). MicroRNAs in cancer. Annu. Rev. Pathol. *4*, 199–227.

Lee, B.-H., Park, S.-Y., Kang, K.-B., Park, R.-W., and Kim, I.-S. (2002). NF-kappaB activates fibronectin gene expression in rat hepatocytes. Biochem. Biophys. Res. Commun. *297*, 1218–1224.

Lee, C.-P., Liu, P.-T., Kung, H.-N., Su, M.-T., Chua, H.-H., Chang, Y.-H., Chang, C.-W., Tsai, C.-H., Liu, F.-T., and Chen, M.-R. (2012). The ESCRT machinery is recruited by the viral BFRF1 protein to the nucleus-associated membrane for the maturation of Epstein-Barr Virus. PLoS Pathog. *8*, e1002904.

Lee, E.-W., Lee, M.-S., Camus, S., Ghim, J., Yang, M.-R., Oh, W., Ha, N.-C., Lane, D.P., and Song, J. (2009a). Differential regulation of p53 and p21 by MKRN1 E3 ligase controls cell cycle arrest and apoptosis. EMBO J. *28*, 2100–2113.

Lee, J.-A., Beigneux, A., Ahmad, S.T., Young, S.G., and Gao, F.-B. (2007). ESCRT-III dysfunction causes autophagosome accumulation and neurodegeneration. Curr. Biol. *17*, 1561–1567.

Lee, S.-J., Lim, H.-S., Masliah, E., and Lee, H.-J. (2011). Protein aggregate spreading in neurodegenerative diseases: problems and perspectives. Neurosci. Res. *70*, 339–348.

Lee, Y., Pressman, S., Andress, A., and Kim, K. (2009b). Silencing by small RNAs is linked to endosomal trafficking. Nat. Cell Biol. *11*, 1150–1156.

Leithe, E., Kjenseth, A., Sirnes, S., Stenmark, H., Brech, A., and Rivedal, E. (2009). Ubiquitylation of the gap junction protein connexin-43 signals its trafficking from early endosomes to lysosomes in a process mediated by Hrs and Tsg101. J. Cell Sci. *122*, 3883–

3893.

Li, L., and Cohen, S.N. (1996). tsg101: A Novel Tumor Susceptibility Gene Isolated by Controlled Homozygous Functional Knockout of Allelic Loci in Mammalian Cells. Cell *85*, 319–329.

Li, Z., and Blissard, G.W. (2012). Cellular VPS4 is required for efficient entry and egress of budded virions of Autographa californica multiple nucleopolyhedrovirus. J. Virol. *86*, 459–472.

Li, J., Belogortseva, N., Porter, D., and Park, M. (2008). Chmp1A functions as a novel tumor suppressor gene in human embryonic kidney and ductal pancreatic tumor cells. Cell Cycle *7*, 2886–2893.

Li, K., Liu, J., Tian, M., Gao, G., Qi, X., Pan, Y., Ruan, J., Liu, C., and Su, X. (2015). CHMP4C Disruption Sensitizes the Human Lung Cancer Cells to Irradiation. Int. J. Mol. Sci. *17*.

Lin, Y.-S., Chen, Y.-J., Cohen, S.N., and Cheng, T.-H. (2013). Identification of TSG101 functional domains and p21 loci required for TSG101-mediated p21 gene regulation. PLoS One *8*, e79674.

Lindås, A.-C., Karlsson, E.A., Lindgren, M.T., Ettema, T.J.G., and Bernander, R. (2008). A unique cell division machinery in the Archaea. Proc. Natl. Acad. Sci. U. S. A. *105*, 18942–18946.

Liu, D., Yang, Z., and Jiang, S. (2011). Identification of PEG10 and TSG101 as carcinogenesis, progression, and poor-prognosis related biomarkers for gallbladder adenocarcinoma. Pathol. Oncol. Res. *17*, 859–866.

Liu, F., Yu, Y., Jin, Y., and Fu, S. (2010). TSG101, identified by screening a cancer cDNA library and soft agar assay, promotes cell proliferation in human lung cancer. Mol. Biol. Rep. *37*, 2829–2838.

Liu, H., Jang, J.K., Kato, N., and McKim, K.S. (2002a). mei-P22 encodes a chromosome-associated protein required for the initiation of meiotic recombination in Drosophila melanogaster. Genetics *162*, 245–258.

Liu, R.-T., Huang, C.-C., You, H.-L., Chou, F.-F., Hu, C.-C.A., Chao, F.-P., Chen, C.-M., and Cheng, J.-T. (2002b). Overexpression of tumor susceptibility gene TSG101 in human papillary thyroid carcinomas. Oncogene *21*, 4830–4837.

Lloyd, T.E., Atkinson, R., Wu, M.N., Zhou, Y., Pennetta, G., and Bellen, H.J. (2002). Hrs Regulates Endosome Membrane Invagination and Tyrosine Kinase Receptor Signaling in Drosophila. Cell *108*, 261–269.

Lobert, V.H., and Stenmark, H. (2011). Cell polarity and migration: emerging role for the endosomal sorting machinery. Physiology (Bethesda). *26*, 171–180.

Logarinho, E., Bousbaa, H., Dias, J.M., Lopes, C., Amorim, I., Antunes-Martins, A., and Sunkel, C.E. (2004). Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in Drosophila cells. J. Cell Sci. *117*.

Loncle, N., Agromayor, M., Martin-Serrano, J., and Williams, D.W. (2015). An ESCRT module is required for neuron pruning. Sci. Rep. *5*, 8461.

Lu, H., and Bilder, D. (2005). Endocytic control of epithelial polarity and proliferation in Drosophila. Nat. Cell Biol. *7*, 1232–1239.

Lund, V.K., and Delotto, R. (2011). Regulation of Toll and Toll-like receptor signaling by the endocytic pathway. Small GTPases *2*, 95–98.

Luo, H., and Dearolf, C.R. (2001). The JAK/STAT pathway andDrosophila development. BioEssays *23*, 1138–1147.

Luo, H. -z., Zhou, Z., Yang, L., Yu, Y., Tian, C., Zhou, B., Zheng, X.-L., Xia, Q., Li, Y., and Wang, R. (2005). Clinicopathologic and Prognostic Significance of MMP-7 (Matrilysin) Expression in Human Rectal Cancer. Jpn. J. Clin. Oncol. *35*, 739–744.

MacDonald, C., Payne, J.A., Aboian, M., Smith, W., Katzmann, D.J., and Piper, R.C. (2015). A family of tetraspans organizes cargo for sorting into multivesicular bodies. Dev. Cell *33*, 328–342.

Madison, M.N., and Okeoma, C.M. (2015). Exosomes: Implications in HIV-1 Pathogenesis. Viruses *7*, 4093–4118.

Mageswaran, S.K., Dixon, M.G., Curtiss, M., Keener, J.P., and Babst, M. (2014). Binding to any ESCRT can mediate ubiquitin-independent cargo sorting. Traffic *15*, 212–229.

Majumdar, I., Ahuja, V., and Paul, J. (2017). Altered expression of Tumor Necrosis Factor Alpha -Induced Protein 3 correlates with disease severity in Ulcerative Colitis. Sci. Rep. *7*, 9420.

Malerød, L., and Stenmark, H. (2009). ESCRTing membrane deformation. Cell *136*, 15–17.

Mamińska, A., Bartosik, A., Banach-Orłowska, M., Pilecka, I., Jastrzębski, K., Zdżalik-Bielecka, D., Castanon, I., Poulain, M., Neyen, C., Wolińska-Nizioł, L., et al. (2016). ESCRT proteins restrict constitutive NF-κB signaling by trafficking cytokine receptors. Sci. Signal. *9*, ra8-ra8.

Martinelli, N., Hartlieb, B., Usami, Y., Sabin, C., Dordor, A., Miguet, N., Avilov, S.

V, Ribeiro, E.A., Göttlinger, H., and Weissenhorn, W. (2012). CC2D1A is a regulator of ESCRT-III CHMP4B. J. Mol. Biol. *419*, 75–88.

Matsuno, K., Diederich, R.J., Go, M.J., Blaumueller, C.M., and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. Development *121*, 2633–2644.

Matusek, T., Wendler, F., Polès, S., Pizette, S., D'Angelo, G., Fürthauer, M., and Thérond, P.P. (2014). The ESCRT machinery regulates the secretion and long-range activity of Hedgehog. Nature *516*, 99–103.

Mazaleyrat, S.L., Fostier, M., Wilkin, M.B., Aslam, H., Evans, D.A.P., Cornell, M., and Baron, M. (2003). Down-regulation of Notch target gene expression by Suppressor of deltex. Dev. Biol. *255*, 363–372.

McCullough, J., Colf, L.A., and Sundquist, W.I. (2013). Membrane fission reactions of the mammalian ESCRT pathway. Annu. Rev. Biochem. *82*, 663–692.

McKay, H.F., and Burgess, D.R. (2011). "Life is a Highway": Membrane Trafficking During Cytokinesis. Traffic *12*, 247–251.

Meckes, D.G., Shair, K.H.Y., Marquitz, A.R., Kung, C.-P., Edwards, R.H., and Raab-Traub, N. (2010). Human tumor virus utilizes exosomes for intercellular communication. Proc. Natl. Acad. Sci. U. S. A. *107*, 20370–20375.

Mehra, A., Zahra, A., Thompson, V., Sirisaengtaksin, N., Wells, A., Porto, M., Köster, S., Penberthy, K., Kubota, Y., Dricot, A., et al. (2013). Mycobacterium tuberculosis Type VII Secreted Effector EsxH Targets Host ESCRT to Impair Trafficking. PLoS Pathog. *9*, e1003734.

Melnikova, L., Biessmann, H., and Georgiev, P. (2005). The Ku protein complex is involved in length regulation of Drosophila telomeres. Genetics *170*, 221–235.

Meng, B., Ip, N.C.Y., Prestwood, L.J., Abbink, T.E.M., and Lever, A.M.L. (2015). Evidence that the endosomal sorting complex required for transport-II (ESCRT-II) is required for efficient human immunodeficiency virus-1 (HIV-1) production. Retrovirology *12*, 72.

Miles, W.O., Dyson, N.J., and Walker, J.A. (2011). Modeling tumor invasion and metastasis in Drosophila. Dis. Model. Mech. *4*, 753–761.

Min, B., Weinert, B.T., and Rio, D.C. (2004). Interplay between Drosophila Bloom's syndrome helicase and Ku autoantigen during nonhomologous end joining repair of P element-induced DNA breaks. Proc. Natl. Acad. Sci. U. S. A. *101*, 8906–8911.

Moberg, K., Schelble, S., Burdick, S., and Hariharan, I. (2005a). Mutations in erupted, the Drosophila Ortholog of Mammalian Tumor Susceptibility Gene 101, Elicit Non-

Cell-Autonomous Overgrowth. Dev. Cell *9*, 699–710.

Moberg, K.H., Schelble, S., Burdick, S.K., and Hariharan, I.K. (2005b). Mutations in erupted, the Drosophila ortholog of mammalian tumor susceptibility gene 101, elicit non-cell-autonomous overgrowth. Dev. Cell *9*, 699–710.

Mochida, G.H., Ganesh, V.S., de Michelena, M.I., Dias, H., Atabay, K.D., Kathrein, K.L., Huang, H.-T., Hill, R.S., Felie, J.M., Rakiec, D., et al. (2012). CHMP1A encodes an essential regulator of BMI1-INK4A in cerebellar development. Nat. Genet. *44*, 1260–1264.

Morawa, K.S., Schneider, M., and Klein, T. (2015). Lgd regulates the activity of the BMP/Dpp signalling pathway during Drosophila oogenesis. Development *142*, 1325–1335.

Morelli, E., Ginefra, P., Mastrodonato, V., Beznoussenko, G. V, Rusten, T.E., Bilder, D., Stenmark, H., Mironov, A.A., and Vaccari, T. (2014). Multiple functions of the SNARE protein Snap29 in autophagy, endocytic, and exocytic trafficking during epithelial formation in Drosophila. Autophagy *10*, 2251–2268.

Morelli, E., Mastrodonato, V., Beznoussenko, G. V, Mironov, A.A., Tognon, E., and Vaccari, T. (2016). An essential step of kinetochore formation controlled by the SNARE protein Snap29. EMBO J. *35*, 2223–2237.

Morita, E., Colf, L.A., Karren, M.A., Sandrin, V., Rodesch, C.K., and Sundquist, W.I. (2010). Human ESCRT-III and VPS4 proteins are required for centrosome and spindle maintenance. Proc. Natl. Acad. Sci. U. S. A. *107*, 12889–12894.

Morita, E., Sandrin, V., McCullough, J., Katsuyama, A., Baci Hamilton, I., and Sundquist, W.I. (2011). ESCRT-III protein requirements for HIV-1 budding. Cell Host Microbe *9*, 235–242.

Mosimann, C., Hausmann, G., and Basler, K. (2006). Parafibromin/Hyrax activates Wnt/Wg target gene transcription by direct association with beta-catenin/Armadillo. Cell *125*, 327–341.

Mostafavi, S., Ray, D., Warde-Farley, D., Grouios, C., and Morris, Q. (2008). GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function. Genome Biol. *9 Suppl 1*, S4.

Mrschtik, M., and Ryan, K.M. (2015). Lysosomal proteins in cell death and autophagy. FEBS J. *282*, 1858–1870.

Mukherjee, A., Veraksa, A., Bauer, A., Rosse, C., Camonis, J., and Artavanis-Tsakonas, S. (2005). Regulation of Notch signalling by non-visual beta-arrestin. Nat. Cell Biol. *7*, 1191–1201.

Müller, M., Schmidt, O., Angelova, M., Faserl, K., Weys, S., Kremser, L.,

Pfaffenwimmer, T., Dalik, T., Kraft, C., Trajanoski, Z., et al. (2015). The coordinated action of the MVB pathway and autophagy ensures cell survival during starvation. Elife *4*, e07736.

Myllykangas, L., Tyynelä, J., Page-McCaw, A., Rubin, G.M., Haltia, M.J., and Feany, M.B. (2005). Cathepsin D-deficient Drosophila recapitulate the key features of neuronal ceroid lipofuscinoses. Neurobiol. Dis. *19*, 194–199.

Nabhan, J.F., Hu, R., Oh, R.S., Cohen, S.N., and Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. Proc. Natl. Acad. Sci. U. S. A. *109*, 4146–4151.

Narayanan, A., Iordanskiy, S., Das, R., Van Duyne, R., Santos, S., Jaworski, E., Guendel, I., Sampey, G., Dalby, E., Iglesias-Ussel, M., et al. (2013). Exosomes derived from HIV-1-infected cells contain trans-activation response element RNA. J. Biol. Chem. *288*, 20014–20033.

Neto, H., Kaupisch, A., Collins, L.L., and Gould, G.W. (2013). Syntaxin 16 is a master recruitment factor for cytokinesis. Mol. Biol. Cell *24*, 3663–3674.

Nielsen, T.T., Mizielinska, S., Hasholt, L., Isaacs, A.M., and Nielsen, J.E. (2012). Reversal of pathology in CHMP2B-mediated frontotemporal dementia patient cells using RNA interference. J. Gene Med. *14*, 521–529.

Norton, P.A., Reis, H.M.G.P. V., Prince, S., Larkin, J., Pan, J., Liu, J., Gong, Q., Zhu, M., and Feitelson, M.A. (2004). Activation of fibronectin gene expression by hepatitis B virus x antigen. J. Viral Hepat. *11*, 332–341.

Obita, T., Saksena, S., Ghazi-Tabatabai, S., Gill, D.J., Perisic, O., Emr, S.D., and Williams, R.L. (2007). Structural basis for selective recognition of ESCRT-III by the AAA ATPase Vps4. Nature *449*, 735–739.

Oh, K.B., Stanton, M.J., West, W.W., Todd, G.L., and Wagner, K.-U. (2007). Tsg101 is upregulated in a subset of invasive human breast cancers and its targeted overexpression in transgenic mice reveals weak oncogenic properties for mammary cancer initiation. Oncogene *26*, 5950–5959.

Okajima, K., Korotchkina, L.G., Prasad, C., Rupar, T., Phillips III, J.A., Ficicioglu, C., Hertecant, J., Patel, M.S., and Kerr, D.S. (2008). Mutations of the E1β subunit gene (PDHB) in four families with pyruvate dehydrogenase deficiency. Mol. Genet. Metab. *93*, 371–380.

Olmos, Y., Hodgson, L., Mantell, J., Verkade, P., and Carlton, J.G. (2015). ESCRT-III controls nuclear envelope reformation. Nature *522*, 236–239.

Omwancha, J., Zhou, X.-F., Chen, S.-Y., Baslan, T., Fisher, C.J., Zheng, Z., Cai, C., and Shemshedini, L. (2006). Makorin RING Finger Protein 1 (MKRN1) Has Negative and Positive Effects on RNA Polymerase II-Dependent Transcription. Endocrine *29*, 363–374.

Overstreet, E., Fitch, E., and Fischer, J.A. (2004). Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. Development *131*, 5355–5366.

Palacios, F., Tushir, J.S., Fujita, Y., and D'Souza-Schorey, C. (2005). Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. Mol. Cell. Biol. *25*, 389–402.

Papadopoulos, C., Orso, G., Mancuso, G., Herholz, M., Gumeni, S., Tadepalle, N., Jüngst, C., Tzschichholz, A., Schauss, A., Höning, S., et al. (2015). Spastin binds to lipid droplets and affects lipid metabolism. PLoS Genet. *11*, e1005149.

Park, S.H., Zhu, P.-P., Parker, R.L., and Blackstone, C. (2010). Hereditary spastic paraplegia proteins REEP1, spastin, and atlastin-1 coordinate microtubule interactions with the tubular ER network. J. Clin. Invest. *120*, 1097–1110.

Parkinson, N., Ince, P.G., Smith, M.O., Highley, R., Skibinski, G., Andersen, P.M., Morrison, K.E., Pall, H.S., Hardiman, O., Collinge, J., et al. (2006). ALS phenotypes with mutations in CHMP2B (charged multivesicular body protein 2B). Neurology *67*, 1074–1077.

Pasqual, G., Rojek, J.M., Masin, M., Chatton, J.-Y., and Kunz, S. (2011). Old world arenaviruses enter the host cell via the multivesicular body and depend on the endosomal sorting complex required for transport. PLoS Pathog. *7*, e1002232.

Pawliczek, T., and Crump, C.M. (2009). Herpes simplex virus type 1 production requires a functional ESCRT-III complex but is independent of TSG101 and ALIX expression. J. Virol. *83*, 11254–11264.

Pegtel, D.M., Cosmopoulos, K., Thorley-Lawson, D.A., van Eijndhoven, M.A.J., Hopmans, E.S., Lindenberg, J.L., de Gruijl, T.D., Würdinger, T., and Middeldorp, J.M. (2010). Functional delivery of viral miRNAs via exosomes. Proc. Natl. Acad. Sci. U. S. A. *107*, 6328–6333.

Peters, J., Rittger, A., Weisner, R., Knabbe, J., Zunke, F., Rothaug, M., Damme, M., Berkovic, S.F., Blanz, J., Saftig, P., et al. (2015). Lysosomal integral membrane protein type-2 (LIMP-2/SCARB2) is a substrate of cathepsin-F, a cysteine protease mutated in type-B-Kufs-disease. Biochem. Biophys. Res. Commun. *457*, 334–340.

Philips, J.A., Porto, M.C., Wang, H., Rubin, E.J., and Perrimon, N. (2008). ESCRT factors restrict mycobacterial growth. Proc. Natl. Acad. Sci. U. S. A. *105*, 3070–3075.

Piao, X., Kobayashi, T., Wang, L., Shiono, M., Takagi, Y., Sun, G., Abe, M., Hagiwara, Y., Zhang, D., Okimoto, K., et al. (2009). Regulation of folliculin (the BHD gene product) phosphorylation by Tsc2-mTOR pathway. Biochem. Biophys. Res. Commun. *389*, 16–21.

Polo, S. (2012). Signaling-mediated control of ubiquitin ligases in endocytosis.

Pornillos, O., Alam, S.L., Davis, D.R., and Sundquist, W.I. (2002). Structure of the Tsg101 UEV domain in complex with the PTAP motif of the HIV-1 p6 protein. Nat. Struct. Biol. *9*, 812–817.

Prescher, J., Baumgärtel, V., Ivanchenko, S., Torrano, A.A., Bräuchle, C., Müller, B., and Lamb, D.C. (2015). Super-resolution imaging of ESCRT-proteins at HIV-1 assembly sites. PLoS Pathog. *11*, e1004677.

Pursiheimo, J.-P., Rantanen, K., Heikkinen, P.T., Johansen, T., and Jaakkola, P.M. (2009). Hypoxia-activated autophagy accelerates degradation of SQSTM1/p62. Oncogene *28*, 334–344.

Qiu, L., Joazeiro, C., Fang, N., Wang, H., Elly, C., Altman, Y., Fang, D., Hunter, T., and Liu, Y. (2000). Recognition and Ubiquitination of Notch by Itch , a Hect-type E3 Ubiquitin Ligase * and promotes ubiquitination of Notch through its Hect. *275*, 35734–35737.

Raab, M., Gentili, M., de Belly, H., Thiam, H.R., Vargas, P., Jimenez, A.J., Lautenschlaeger, F., Voituriez, R., Lennon-Duménil, A.M., Manel, N., et al. (2016). ESCRT III repairs nuclear envelope ruptures during cell migration to limit DNA damage and cell death. Science *352*, 359–362.

Raiborg, C., and Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature *458*, 445–452.

Raiborg, C., Bache, K.G., Mehlum, A., Stang, E., and Stenmark, H. (2001). Hrs recruits clathrin to early endosomes. EMBO J. *20*, 5008–5021.

Raiborg, C., Bache, K.G., Gillooly, D.J., Madshus, I.H., Stang, E., and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. Nat. Cell Biol. *4*, 394–398.

Raja, S.J., Charapitsa, I., Conrad, T., Vaquerizas, J.M., Gebhardt, P., Holz, H., Kadlec, J., Fraterman, S., Luscombe, N.M., and Akhtar, A. (2010). The Nonspecific Lethal Complex Is a Transcriptional Regulator in Drosophila. Mol. Cell *38*, 827–841.

Rath, U., Wang, D., Ding, Y., Xu, Y.-Z., Qi, H., Blacketer, M.J., Girton, J., Johansen, J., and Johansen, K.M. (2004). Chromator, a novel and essential chromodomain protein

interacts directly with the putative spindle matrix protein skeletor. J. Cell. Biochem. *93*, 1033–1047.

Rath, U., Ding, Y., Deng, H., Qi, H., Bao, X., Zhang, W., Girton, J., Johansen, J., and Johansen, K.M. (2006). The chromodomain protein, Chromator, interacts with JIL-1 kinase and regulates the structure of Drosophila polytene chromosomes. J. Cell Sci. *119*, 2332–2341.

Raymond, C.K., Howald-Stevenson, I., Vater, C.A., and Stevens, T.H. (1992). Morphological classification of the yeast vacuolar protein sorting mutants: evidence for a prevacuolar compartment in class E vps mutants. Mol. Biol. Cell *3*, 1389–1402.

Reid, E., Connell, J., Edwards, T.L., Duley, S., Brown, S.E., and Sanderson, C.M. (2005). The hereditary spastic paraplegia protein spastin interacts with the ESCRT-III complex-associated endosomal protein CHMP1B. Hum. Mol. Genet. *14*, 19–38.

Ren, A., Zhang, W., Yarlagadda, S., Sinha, C., Arora, K., Moon, C.-S., and Naren, A.P. (2013). MAST205 Competes with Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-associated Ligand for Binding to CFTR to Regulate CFTR-mediated Fluid Transport. J. Biol. Chem. *288*, 12325–12334.

Rimawi, M.F., Shetty, P.B., Weiss, H.L., Schiff, R., Osborne, C.K., Chamness, G.C., and Elledge, R.M. (2010). Epidermal growth factor receptor expression in breast cancer association with biologic phenotype and clinical outcomes. Cancer *116*, 1234–1242.

Robinson, D.R., Kalyana-Sundaram, S., Wu, Y.-M., Shankar, S., Cao, X., Ateeq, B., Asangani, I. a, Iyer, M., Maher, C. a, Grasso, C.S., et al. (2011). Functionally recurrent rearrangements of the MAST kinase and Notch gene families in breast cancer. Nat. Med. *17*, 1646–1651.

Roczniak-Ferguson, A., Petit, C.S., Froehlich, F., Qian, S., Ky, J., Angarola, B., Walther, T.C., and Ferguson, S.M. (2012). The transcription factor TFEB links mTORC1 signaling to transcriptional control of lysosome homeostasis. Sci. Signal. *5*, ra42.

Rodahl, L.M., Haglund, K., Sem-Jacobsen, C., Wendler, F., Vincent, J.-P., Lindmo, K., Rusten, T.E., and Stenmark, H. (2009). Disruption of Vps4 and JNK function in Drosophila causes tumour growth. PLoS One *4*, e4354.

Rodriguez-Magadán, H., Merino, E., Schnabel, D., Ramírez, L., and Lomelí, H. (2008). Spatial and temporal expression of Zimp7 and Zimp10 PIAS-like proteins in the developing mouse embryo. Gene Expr. Patterns *8*, 206–213.

Rogov, V., Dötsch, V., Johansen, T., and Kirkin, V. (2014). Interactions between Autophagy Receptors and Ubiquitin-like Proteins Form the Molecular Basis for Selective

Autophagy. Mol. Cell *53*, 167–178.

Rosen, D.B., Putta, S., Covey, T., Huang, Y.-W., Nolan, G.P., Cesano, A., Minden, M.D., and Fantl, W.J. (2010). Distinct Patterns of DNA Damage Response and Apoptosis Correlate with Jak/Stat and PI3Kinase Response Profiles in Human Acute Myelogenous Leukemia. PLoS One *5*, e12405.

Rothman, J.H., Howald, I., and Stevens, T.H. (1989). Characterization of genes required for protein sorting and vacuolar function in the yeast Saccharomyces cerevisiae. EMBO J. *8*, 2057–2065.

Roudier, N., Lefebvre, C., and Legouis, R. (2005). CeVPS-27 is an endosomal protein required for the molting and the endocytic trafficking of the low-density lipoprotein receptor-related protein 1 in Caenorhabditis elegans. Traffic *6*, 695–705.

Ruland, J., Sirard, C., Elia, A., MacPherson, D., Wakeham, A., Li, L., de la Pompa, J.L., Cohen, S.N., and Mak, T.W. (2001). p53 accumulation, defective cell proliferation, and early embryonic lethality in mice lacking tsg101. Proc. Natl. Acad. Sci. U. S. A. *98*, 1859–1864.

Rusten, T.E., and Simonsen, A. (2008). ESCRT functions in autophagy and associated disease. Cell Cycle *7*, 1166–1172.

Rusten, T.E., Vaccari, T., Lindmo, K., Rodahl, L.M.W., Nezis, I.P., Sem-Jacobsen, C., Wendler, F., Vincent, J.-P., Brech, A., Bilder, D., et al. (2007). ESCRTs and Fab1 regulate distinct steps of autophagy. Curr. Biol. *17*, 1817–1825.

Rusten, T.E., Vaccari, T., and Stenmark, H. (2012). Shaping development with ESCRTs. Nat. Cell Biol. *14*, 38–45.

Safaei, R., Larson, B.J., Cheng, T.C., Gibson, M.A., Otani, S., Naerdemann, W., and Howell, S.B. (2005). Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells. Mol. Cancer Ther. *4*, 1595–1604.

Sagona, A.P., Nezis, I.P., and Stenmark, H. (2014). Association of CHMP4B and autophagy with micronuclei: implications for cataract formation. Biomed Res. Int. *2014*, 974393.

Sahu, R., Kaushik, S., Clement, C.C., Cannizzo, E.S., Scharf, B., Follenzi, A., Potolicchio, I., Nieves, E., Cuervo, A.M., and Santambrogio, L. (2011). Microautophagy of cytosolic proteins by late endosomes. Dev. Cell *20*, 131–139.

Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K., and Hayashi, S. (2004). Drosophila Nedd4 Regulates Endocytosis of Notch and Suppresses Its Ligand-Independent Activation. Curr. Biol. *14*, 2228–2236.

Salvatico, J., Kim, J.H., Chung, I.K., and Muller, M.T. (2010). Differentiation linked regulation of telomerase activity by Makorin-1. Mol. Cell. Biochem. *342*, 241–250.

Samson, R.Y., and Bell, S.D. (2009). Ancient ESCRTs and the evolution of binary fission. Trends Microbiol. *17*, 507–513.

Sandrin, V., and Sundquist, W.I. (2013). ESCRT requirements for EIAV budding. Retrovirology *10*, 104.

Sang, T.-K., and Jackson, G.R. (2005). Drosophila models of neurodegenerative disease. NeuroRx *2*, 438–446.

Sato, K., Miyahara, M., Saito, T., and Kobayashi, M. (1994). c-myc mRNA overexpression is associated with lymph node metastasis in colorectal cancer. Eur. J. Cancer *30*, 1113–1117.

Schmidt, A.E., Miller, T., Schmidt, S.L., Shiekhattar, R., and Shilatifard, A. (1999). Cloning and characterization of the EAP30 subunit of the ELL complex that confers derepression of transcription by RNA polymerase II. J. Biol. Chem. *274*, 21981–21985.

Schneider, M., Troost, T., Grawe, F., Martinez-Arias, A., and Klein, T. (2013). Activation of Notch in lgd mutant cells requires the fusion of late endosomes with the lysosome. J Cell Sci *126*, 645–656.

Seto, E.S., and Bellen, H.J. (2006). Internalization is required for proper Wingless signaling in Drosophila melanogaster. J. Cell Biol. *173*, 95–106.

Settembre, C., Di Malta, C., Polito, V.A., Garcia Arencibia, M., Vetrini, F., Erdin, S., Erdin, S.U., Huynh, T., Medina, D., Colella, P., et al. (2011). TFEB links autophagy to lysosomal biogenesis. Science *332*, 1429–1433.

Settembre, C., Zoncu, R., Medina, D.L., Vetrini, F., Erdin, S., Erdin, S., Huynh, T., Ferron, M., Karsenty, G., Vellard, M.C., et al. (2012). A lysosome-to-nucleus signalling mechanism senses and regulates the lysosome via mTOR and TFEB. EMBO J. *31*, 1095–1108.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. *13*, 2498–2504.

Shi, J., Wang, E., Milazzo, J.P., Wang, Z., Kinney, J.B., and Vakoc, C.R. (2015). Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. Nat. Biotechnol. *33*, 661–667.

Shields, S.B., and Piper, R.C. (2011). How ubiquitin functions with ESCRTs. Traffic *12*, 1306–1317.

Shields, S.B., Oestreich, A.J., Winistorfer, S., Nguyen, D., Payne, J. a, Katzmann, D.J., and Piper, R. (2009). ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting. J. Cell Biol. *185*, 213–224.

Shiels, A., Bennett, T.M., Knopf, H.L.S., Yamada, K., Yoshiura, K., Niikawa, N., Shim, S., and Hanson, P.I. (2007). CHMP4B, a novel gene for autosomal dominant cataracts linked to chromosome 20q. Am. J. Hum. Genet. *81*, 596–606.

Shim, J.-H., Xiao, C., Hayden, M.S., Lee, K.-Y., Trombetta, E.S., Pypaert, M., Nara, A., Yoshimori, T., Wilm, B., Erdjument-Bromage, H., et al. (2006). CHMP5 is essential for late endosome function and down-regulation of receptor signaling during mouse embryogenesis. J. Cell Biol. *172*, 1045–1056.

Shtanko, O., Nikitina, R.A., Altuntas, C.Z., Chepurnov, A.A., and Davey, R.A. (2014). Crimean-Congo hemorrhagic fever virus entry into host cells occurs through the multivesicular body and requires ESCRT regulators. PLoS Pathog. *10*, e1004390.

Sigismund, S., Confalonieri, S., Ciliberto, A., Polo, S., Scita, G., and Di Fiore, P.P. (2012). Endocytosis and signaling: cell logistics shape the eukaryotic cell plan. Physiol. Rev. *92*, 273–366.

Silva-Ayala, D., López, T., Gutiérrez, M., Perrimon, N., López, S., and Arias, C.F. (2013). Genome-wide RNAi screen reveals a role for the ESCRT complex in rotavirus cell entry. Proc. Natl. Acad. Sci. U. S. A. *110*, 10270–10275.

Silver-Morse, L., and Li, W.X. (2013). JAK-STAT in heterochromatin and genome stability. JAK-STAT *2*, e26090.

Silvestri, L.S., Ruthel, G., Kallstrom, G., Warfield, K.L., Swenson, D.L., Nelle, T., Iversen, P.L., Bavari, S., and Aman, M.J. (2007). Involvement of vacuolar protein sorting pathway in Ebola virus release independent of TSG101 interaction. J. Infect. Dis. *196 Suppl*, S264-70.

Simon, M., Johansson, C., and Mirazimi, A. (2009). Crimean-Congo hemorrhagic fever virus entry and replication is clathrin-, pH- and cholesterol-dependent. J. Gen. Virol. *90*, 210–215.

Singh, J., Aaronson, S.A., and Mlodzik, M. (2010). Drosophila Abelson kinase mediates cell invasion and proliferation through two distinct MAPK pathways. Oncogene *29*, 4033–4045.

Skibinski, G., Parkinson, N.J., Brown, J.M., Chakrabarti, L., Lloyd, S.L., Hummerich, H., Nielsen, J.E., Hodges, J.R., Spillantini, M.G., Thusgaard, T., et al. (2005). Mutations in the endosomal ESCRTIII-complex subunit CHMP2B in frontotemporal dementia. Nat.

Genet. *37*, 806–808.

Slagsvold, T., Aasland, R., Hirano, S., Bache, K.G., Raiborg, C., Trambaiolo, D., Wakatsuki, S., and Stenmark, H. (2005). Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. J. Biol. Chem. *280*, 19600–19606.

Small, D.M., Morais, C., Coombes, J.S., Bennett, N.C., Johnson, D.W., and Gobe, G.C. (2014). Oxidative stress-induced alterations in PPAR-γ and associated mitochondrial destabilization contribute to kidney cell apoptosis. Am. J. Physiol. - Ren. Physiol. *307*.

Smith, K.R., Dahl, H.H.M., Canafoglia, L., Andermann, E., Damiano, J., Morbin, M., Bruni, A.C., Giaccone, G., Cossette, P., Saftig, P., et al. (2013). Cathepsin F mutations cause Type B Kufs disease, an adult-onset neuronal ceroid lipofuscinosis. Hum. Mol. Genet. *22*, 1417–1423.

Son, J.H., Shim, J.H., Kim, K.-H., Ha, J.-Y., and Han, J.Y. (2012). Neuronal autophagy and neurodegenerative diseases. Exp. Mol. Med. *44*, 89.

Sorkin, A., and von Zastrow, M. (2009). Endocytosis and signalling: intertwining molecular networks. Nat. Rev. Mol. Cell Biol. *10*, 609–622.

Spencer, B., Emadi, S., Desplats, P., Eleuteri, S., Michael, S., Kosberg, K., Shen, J., Rockenstein, E., Patrick, C., Adame, A., et al. (2014). ESCRT-mediated uptake and degradation of brain-targeted α-synuclein single chain antibody attenuates neuronal degeneration in vivo. Mol. Ther. *22*, 1753–1767.

Spencer, B., Kim, C., Gonzalez, T., Bisquertt, A., Patrick, C., Rockenstein, E., Adame, A., Lee, S.-J., Desplats, P., and Masliah, E. (2016). α-Synuclein interferes with the ESCRT-III complex contributing to the pathogenesis of Lewy Body disease. Hum. Mol. Genet. *25*, 1100–1115.

Stack, J.H., and Emr, S.D. (1993). Genetic and biochemical studies of protein sorting to the yeast vacuole. Curr. Opin. Cell Biol. *5*, 641–646.

Stauffer, D.R., Howard, T.L., Nyun, T., and Hollenberg, S.M. (2001). CHMP1 is a novel nuclear matrix protein affecting chromatin structure and cell-cycle progression. J. Cell Sci. *114*, 2383–2393.

Stieler, J.T., and Prange, R. (2014). Involvement of ESCRT-II in hepatitis B virus morphogenesis. PLoS One *9*, e91279.

Stuchell-Brereton, M.D., Skalicky, J.J., Kieffer, C., Karren, M.A., Ghaffarian, S., and Sundquist, W.I. (2007). ESCRT-III recognition by VPS4 ATPases. Nature *449*, 740–744.

Sudo, T., Iwaya, T., Nishida, N., Sawada, G., Takahashi, Y., Ishibashi, M., Shibata, K., Fujita, H., Shirouzu, K., Mori, M., et al. (2013). Expression of Mesenchymal Markers

Vimentin and Fibronectin: The Clinical Significance in Esophageal Squamous Cell Carcinoma. Ann. Surg. Oncol. *20*, 324–335.

Sun, Z., Pan, J., Hope, W.X., Cohen, S.N., and Balk, S.P. (1999). Tumor susceptibility gene 101 protein represses androgen receptor transactivation and interacts with p300. Cancer *86*, 689–696.

Sung, H.-H., Telley, I.A., Papadaki, P., Ephrussi, A., Surrey, T., and Rørth, P. (2008). Drosophila Ensconsin Promotes Productive Recruitment of Kinesin-1 to Microtubules. Dev. Cell *15*, 866–876.

Sutherland, D., Samakovlis, C., and Krasnow, M.A. (1996). branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. Cell *87*, 1091–1101.

Sweeney, N.T., Brenman, J.E., Jan, Y.N., and Gao, F.-B. (2006). The coiled-coil protein shrub controls neuronal morphogenesis in Drosophila. Curr. Biol. *16*, 1006–1011.

Taelman, V.F., Dobrowolski, R., Plouhinec, J.-L., Fuentealba, L.C., Vorwald, P.P., Gumper, I., Sabatini, D.D., and De Robertis, E.M. (2010). Wnt signaling requires sequestration of glycogen synthase kinase 3 inside multivesicular endosomes. Cell *143*, 1136–1148.

Takagi, Y., Kobayashi, T., Shiono, M., Wang, L., Piao, X., Sun, G., Zhang, D., Abe, M., Hagiwara, Y., Takahashi, K., et al. (2008). Interaction of folliculin (Birt-Hogg-Dubé gene product) with a novel Fnip1-like (FnipL/Fnip2) protein. Oncogene *27*, 5339–5347.

Tamai, K., Shiina, M., Tanaka, N., Nakano, T., Yamamoto, A., Kondo, Y., Kakazu, E., Inoue, J., Fukushima, K., Sano, K., et al. (2012). Regulation of hepatitis C virus secretion by the Hrs-dependent exosomal pathway. Virology *422*, 377–385.

Tang, C.-H., Lee, J.-W., Galvez, M.G., Robillard, L., Mole, S.E., and Chapman, H.A. (2006a). Murine cathepsin F deficiency causes neuronal lipofuscinosis and late-onset neurological disease. Mol. Cell. Biol. *26*, 2309–2316.

Tang, C.-H., Lee, J.-W., Galvez, M.G., Robillard, L., Mole, S.E., and Chapman, H.A. (2006b). Murine cathepsin F deficiency causes neuronal lipofuscinosis and late-onset neurological disease. Mol. Cell. Biol. *26*, 2309–2316.

Teis, D., Saksena, S., Judson, B.L., and Emr, S.D. (2010). ESCRT-II coordinates the assembly of ESCRT-III filaments for cargo sorting and multivesicular body vesicle formation. EMBO J. *29*, 871–883.

Temme, S., Eis-Hübinger, A.M., McLellan, A.D., and Koch, N. (2010). The herpes simplex virus-1 encoded glycoprotein B diverts HLA-DR into the exosome pathway. J.

Immunol. *184*, 236–243.

Teo, H., Perisic, O., González, B., and Williams, R.L. (2004). ESCRT-II, an endosome-associated complex required for protein sorting: crystal structure and interactions with ESCRT-III and membranes. Dev. Cell *7*, 559–569.

Teo, H., Gill, D.J., Sun, J., Perisic, O., Veprintsev, D.B., Vallis, Y., Emr, S.D., and Williams, R.L. (2006). ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. Cell *125*, 99–111.

Terrien, E., Chaffotte, A., Lafage, M., Khan, Z., Préhaud, C., Cordier, F., Simenel, C., Delepierre, M., Buc, H., Lafon, M., et al. (2012). Interference with the PTEN-MAST2 Interaction by a Viral Protein Leads to Cellular Relocalization of PTEN. Sci. Signal. *5*, ra58.

Thompson, B.J., Mathieu, J., Sung, H.-H., Loeser, E., Rørth, P., and Cohen, S.M. (2005). Tumor suppressor properties of the ESCRT-II complex component Vps25 in Drosophila. Dev. Cell *9*, 711–720.

Tian, X., Hansen, D., Schedl, T., and Skeath, J.B. (2004). Epsin potentiates Notch pathway activity in Drosophila and C. elegans. Development *131*, 5807–5815.

Tognon, E., Wollscheid, N., Cortese, K., Tacchetti, C., and Vaccari, T. (2014). ESCRT-0 is not required for ectopic Notch activation and tumor suppression in Drosophila. PLoS One *9*, e93987.

Tognon, E., Kobia, F., Busi, I., Fumagalli, A., De Masi, F., and Vaccari, T. (2016). Control of lysosomal biogenesis and Notch-dependent tissue patterning by components of the TFEB-V-ATPase axis in Drosophila melanogaster. Autophagy *12*, 499–514.

Toyoshima, M., Tanaka, N., Aoki, J., Tanaka, Y., Murata, K., Kyuuma, M., Kobayashi, H., Ishii, N., Yaegashi, N., and Sugamura, K. (2007). Inhibition of tumor growth and metastasis by depletion of vesicular sorting protein Hrs: Its regulatory role on E-cadherin and β-catenin. Cancer Res. *67*, 5162–5171.

Troost, T., Jaeckel, S., Ohlenhard, N., and Klein, T. (2012). The tumour suppressor Lethal (2) giant discs is required for the function of the ESCRT-III component Shrub/CHMP4. J. Cell Sci. *125*.

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B., and Turk, D. (2012). Cysteine cathepsins: From structure, function and regulation to new frontiers. Biochim. Biophys. Acta - Proteins Proteomics *1824*, 68–88.

Urbé, S. (2005). Ubiquitin and endocytic protein sorting. Essays Biochem. *41*, 81–98.

Urwin, H., Authier, A., Nielsen, J.E., Metcalf, D., Powell, C., Froud, K., Malcolm, D.S., Holm, I., Johannsen, P., Brown, J., et al. (2010). Disruption of endocytic trafficking in

frontotemporal dementia with CHMP2B mutations. Hum. Mol. Genet. *19*, 2228–2238.

Usami, Y., Popov, S., Weiss, E.R., Vriesema-Magnuson, C., Calistri, A., and Göttlinger, H.G. (2012). Regulation of CHMP4/ESCRT-III function in human immunodeficiency virus type 1 budding by CC2D1A. J. Virol. *86*, 3746–3756.

Vaccari, T., and Bilder, D. (2005). The Drosophila Tumor Suppressor vps25 Prevents Nonautonomous Overproliferation by Regulating Notch Trafficking. Dev. Cell *9*, 687–698.

Vaccari, T., and Bilder, D. (2009). At the crossroads of polarity, proliferation and apoptosis: the use of Drosophila to unravel the multifaceted role of endocytosis in tumor suppression. Mol. Oncol. *3*, 354–365.

Vaccari, T., Lu, H., Kanwar, R., Fortini, M.E., and Bilder, D. (2008). Endosomal entry regulates Notch receptor activation in Drosophila melanogaster. J. Cell Biol. *180*, 755–762.

Vaccari, T., Rusten, T.E., Menut, L., Nezis, I.P., Brech, A., Stenmark, H., and Bilder, D. (2009). Comparative analysis of ESCRT-I, ESCRT-II and ESCRT-III function in Drosophila by efficient isolation of ESCRT mutants. J. Cell Sci. *122*, 2413–2423.

Valiente, M., Andrés-Pons, A., Gomar, B., Torres, J., Gil, A., Tapparel, C., Antonarakis, S.E., and Pulido, R. (2005). Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases. J. Biol. Chem. *280*, 28936–28943.

Vázquez, C.L., and Colombo, M.I. (2009). Chapter 6 Assays to Assess Autophagy Induction and Fusion of Autophagic Vacuoles with a Degradative Compartment, Using Monodansylcadaverine (MDC) and DQ-BSA. In Methods in Enzymology, pp. 85–95.

Vermezovic, J., Adamowicz, M., Santarpia, L., Rustighi, A., Forcato, M., Lucano, C., Massimiliano, L., Costanzo, V., Bicciato, S., Del Sal, G., et al. (2015). Notch is a direct negative regulator of the DNA-damage response. Nat Struct Mol Biol *22*, 417–424.

Vietri, M., Schink, K.O., Campsteijn, C., Wegner, C.S., Schultz, S.W., Christ, L., Thoresen, S.B., Brech, A., Raiborg, C., and Stenmark, H. (2015). Spastin and ESCRT-III coordinate mitotic spindle disassembly and nuclear envelope sealing. Nature *522*, 231–235.

Vincenti, M.P., Coon, C.I., and Brinckerhoff, C.E. (1998). Nuclear factor ?B/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1?-stimulated synovial fibroblasts. Arthritis Rheum. *41*, 1987–1994.

Waalkes, S., Atschekzei, F., Kramer, M.W., Hennenlotter, J., Vetter, G., Becker, J.U., Stenzl, A., Merseburger, A.S., Schrader, A.J., Kuczyk, M.A., et al. (2010). Fibronectin 1 mRNA expression correlates with advanced disease in renal cancer. BMC Cancer *10*, 503.

Wagner, K.-U., Krempler, A., Qi, Y., Park, K., Henry, M.D., Triplett, A.A., Riedlinger, G., Rucker III, E.B., and Hennighausen, L. (2003). Tsg101 is essential for cell growth, proliferation, and cell survival of embryonic and adult tissues. Mol. Cell. Biol. *23*, 150–162.

Walden, P.D., and Cowan, N.J. (1993). A novel 205-kilodalton testis-specific serine/threonine protein kinase associated with microtubules of the spermatid manchette. Mol. Cell. Biol. *13*, 7625–7635.

Walden, P.D., and Millette, C.F. (1996). Increased activity associated with the MAST205 protein kinase complex during mammalian spermiogenesis. Biol. Reprod. *55*, 1039–1044.

Wang, D., Lee, H.J., Cooper, D.S., Cebotaro, L., Walden, P.D., Choi, I., and Yun, C.C. (2006). Coexpression of MAST205 inhibits the activity of Na+/H+ exchanger NHE3. Am. J. Physiol. Renal Physiol. *290*, F428-37.

Wang, P., Galan, J.A., Normandin, K., Bonneil, É., Hickson, G.R., Roux, P.P., Thibault, P., and Archambault, V. (2013). Cell cycle regulation of Greatwall kinase nuclear localization facilitates mitotic progression. J. Cell Biol. *202*, 277–293.

Wang, Y., Zhang, N., Zhang, L., Li, R., Fu, W., Ma, K., Li, X., Wang, L., Wang, J., Zhang, H., et al. (2016). Autophagy Regulates Chromatin Ubiquitination in DNA Damage Response through Elimination of SQSTM1/p62. Mol. Cell *63*, 34–48.

Watson, K.L., Justice, R.W., and Bryant, P.J. (1994). Drosophila in cancer research: the first fifty tumor suppressor genes. J. Cell Sci. Suppl. *18*, 19–33.

Webster, B.M., Colombi, P., Jäger, J., and Lusk, C.P. (2014). Surveillance of nuclear pore complex assembly by ESCRT-III/Vps4. Cell *159*, 388–401.

Wehman, A.M., Poggioli, C., Schweinsberg, P., Grant, B.D., and Nance, J. (2011). The P4-ATPase TAT-5 inhibits the budding of extracellular vesicles in C. elegans embryos. Curr. Biol. *21*, 1951–1959.

Wei, J., Lv, L., Wan, Y., Cao, Y., Li, G., Lin, H., Zhou, R., Shang, C., Cao, J., He, H., et al. (2015). Vps4A functions as a tumor suppressor by regulating the secretion and uptake of exosomal microRNAs in human hepatoma cells. Hepatology *61*, 1284–1294.

Wideman, J.G., Leung, K.F., Field, M.C., and Dacks, J.B. (2014). The cell biology of the endocytic system from an evolutionary perspective. Cold Spring Harb. Perspect. Biol. *6*, a016998.

Wilkin, M., Tongngok, P., Gensch, N., Clemence, S., Motoki, M., Yamada, K., Hori, K., Taniguchi-Kanai, M., Franklin, E., Matsuno, K., et al. (2008). Drosophila HOPS and AP-

3 Complex Genes Are Required for a Deltex-Regulated Activation of Notch in the Endosomal Trafficking Pathway. Dev. Cell *15*, 762–772.

Wilkin, M.B., Carbery, A.-M., Fostier, M., Aslam, H., Mazaleyrat, S.L., Higgs, J., Myat, A., Evans, D.A.P., Cornell, M., and Baron, M. (2004). Regulation of Notch Endosomal Sorting and Signaling by Drosophila Nedd4 Family Proteins. Curr. Biol. *14*, 2237–2244.

Williams, R.L., and Urbé, S. (2007). The emerging shape of the ESCRT machinery. Nat. Rev. Mol. Cell Biol. *8*, 355–368.

Wittinger, M., Vanhara, P., El-Gazzar, A., Savarese-Brenner, B., Pils, D., Anees, M., Grunt, T.W., Sibilia, M., Holcmann, M., Horvat, R., et al. (2011). hVps37A Status affects prognosis and cetuximab sensitivity in ovarian cancer. Clin. Cancer Res. *17*, 7816–7827.

Wolf, J.M., and Davis, D.A. (2010). Mutational analysis of Candida albicans SNF7 reveals genetically separable Rim101 and ESCRT functions and demonstrates divergence in bro1-domain protein interactions. Genetics *184*, 673–694.

Wolf, J.M., Johnson, D.J., Chmielewski, D., and Davis, D.A. (2010). The Candida albicans ESCRT pathway makes Rim101-dependent and -independent contributions to pathogenesis. Eukaryot. Cell *9*, 1203–1215.

Wollert, T., and Hurley, J.H. (2010). Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. Nature *464*, 864–869.

Woodfield, S.E., Graves, H.K., Hernandez, J.A., and Bergmann, A. (2013a). De-regulation of JNK and JAK/STAT signaling in ESCRT-II mutant tissues cooperatively contributes to neoplastic tumorigenesis. PLoS One *8*, e56021.

Woodfield, S.E., Graves, H.K., Hernandez, J. a, and Bergmann, A. (2013b). De-regulation of JNK and JAK/STAT signaling in ESCRT-II mutant tissues cooperatively contributes to neoplastic tumorigenesis. PLoS One *8*, e56021.

Wu, Q.-W., Yang, Q.-M., Huang, Y.-F., She, H.-Q., Liang, J., Yang, Q.-L., and Zhang, Z.-M. (2014). Expression and Clinical Significance of Matrix Metalloproteinase-9 in Lymphatic Invasiveness and Metastasis of Breast Cancer. PLoS One *9*, e97804.

Xie, W., Li, L., and Cohen, S.N. (1998). Cell cycle-dependent subcellular localization of the TSG101 protein and mitotic and nuclear abnormalities associated with TSG101 deficiency. Proc. Natl. Acad. Sci. U. S. A. *95*, 1595–1600.

Xiong, H., Li, H., Chen, Y., Zhao, J., and Unkeless, J.C. (2004). Interaction of TRAF6 with MAST205 regulates NF-kappaB activation and MAST205 stability. J. Biol. Chem. *279*, 43675–43683.

Xu, T., and Artavanis-Tsakonas, S. (1990). deltex, a locus interacting with the

neurogenic genes, Notch, Delta and mastermind in Drosophila melanogaster. Genetics *126*, 665–677.

Xu, F., Li, X., Yan, L., Yuan, N., Fang, Y., Cao, Y., Xu, L., Zhang, X., Xu, L., Ge, C., et al. (2017). Autophagy Promotes the Repair of Radiation-Induced DNA Damage in Bone Marrow Hematopoietic Cells via Enhanced STAT3 Signaling. Radiat. Res. *187*, 382–396.

Xu, J., Yang, W., Wang, Q., Zhang, Q., Li, X., Lin, X., Liu, X., and Qin, Y. (2014). Decreased HCRP1 expression is associated with poor prognosis in breast cancer patients. Int. J. Clin. Exp. Pathol. *7*, 7915–7922.

Xu, W., Smith, F.J., Subaran, R., and Mitchell, A.P. (2004). Multivesicular body-ESCRT components function in pH response regulation in Saccharomyces cerevisiae and Candida albicans. Mol. Biol. Cell *15*, 5528–5537.

Xu, Z., Liang, L., Wang, H., Li, T., and Zhao, M. (2003). HCRP1, a novel gene that is downregulated in hepatocellular carcinoma, encodes a growth-inhibitory protein. Biochem. Biophys. Res. Commun. *311*, 1057–1066.

Yamada, K., Fuwa, T.J., Ayukawa, T., Tanaka, T., Nakamura, A., Wilkin, M.B., Baron, M., and Matsuno, K. (2011). Roles of Drosophila Deltex in Notch receptor endocytic trafficking and activation. Genes to Cells *16*, 261–272.

Yamada, M., Ishii, N., Asao, H., Murata, K., Kanazawa, C., Sasaki, H., and Sugamura, K. (2002). Signal-transducing adaptor molecules STAM1 and STAM2 are required for T-cell development and survival. Mol. Cell. Biol. *22*, 8648–8658.

Yamamoto, A., Cremona, M.L., and Rothman, J.E. (2006). Autophagy-mediated clearance of huntingtin aggregates triggered by the insulin-signaling pathway. J. Cell Biol. *172*, 719–731.

Yang, B., Stjepanovic, G., Shen, Q., Martin, A., and Hurley, J.H. (2015). Vps4 disassembles an ESCRT-III filament by global unfolding and processive translocation. Nat. Struct. Mol. Biol. *22*, 492–498.

Yang, X., Mao, F., Lv, X., Zhang, Z., and Fu, L. (2013). Drosophila Vps36 is involved in Hh signaling by regulating Smo trafficking. J. Cell Sci.

You, K.T., Li, L.S., Kim, N.-G., Kang, H.J., Koh, K.H., Chwae, Y.-J., Kim, K.M., Kim, Y.K., Park, S.M., Jang, S.K., et al. (2007). Selective Translational Repression of Truncated Proteins from Frameshift Mutation-Derived mRNAs in Tumors. PLoS Biol. *5*, e109.

You, Z., Xin, Y., Liu, Y., Sun, J., Zhou, G., Gao, H., Xu, P., Chen, Y., Chen, G.,

Zhang, L., et al. (2012). Chmp1A acts as a tumor suppressor gene that inhibits proliferation of renal cell carcinoma. Cancer Lett. *319*, 190–196.

Young, T.W., Mei, F.C., Rosen, D.G., Yang, G., Li, N., Liu, J., and Cheng, X. (2007a). Up-regulation of tumor susceptibility gene 101 protein in ovarian carcinomas revealed by proteomics analyses. Mol. Cell. Proteomics *6*, 294–304.

Young, T.W., Rosen, D.G., Mei, F.C., Li, N., Liu, J., Wang, X.-F., and Cheng, X. (2007b). Up-regulation of tumor susceptibility gene 101 conveys poor prognosis through suppression of p21 expression in ovarian cancer. Clin. Cancer Res. *13*, 3848–3854.

Yu, J., Fleming, S.L., Williams, B., Williams, E. V., Li, Z., Somma, P., Rieder, C.L., and Goldberg, M.L. (2004). Greatwall kinase. J. Cell Biol. *164*, 487–492.

Zamborlini, A., Usami, Y., Radoshitzky, S.R., Popova, E., Palu, G., and Göttlinger, H. (2006). Release of autoinhibition converts ESCRT-III components into potent inhibitors of HIV-1 budding. Proc. Natl. Acad. Sci. U. S. A. *103*, 19140–19145.

van der Zee, J., Urwin, H., Engelborghs, S., Bruyland, M., Vandenberghe, R., Dermaut, B., De Pooter, T., Peeters, K., Santens, P., De Deyn, P.P., et al. (2008). CHMP2B C-truncating mutations in frontotemporal lobar degeneration are associated with an aberrant endosomal phenotype in vitro. Hum. Mol. Genet. *17*, 313–322.

van der Zee, J., Mariën, P., Crols, R., Van Mossevelde, S., Dillen, L., Perrone, F., Engelborghs, S., Verhoeven, J., D'aes, T., Ceuterick-De Groote, C., et al. (2016). Mutated CTSF in adult-onset neuronal ceroid lipofuscinosis and FTD. Neurol. Genet. *2*, e102.

Zhang, D., Wang, L., Yan, L., Miao, X., Gong, C., Xiao, M., Ni, R., and Tang, Q. (2015a). Vacuolar protein sorting 4B regulates apoptosis of intestinal epithelial cells via p38 MAPK in Crohn's disease. Exp. Mol. Pathol. *98*, 55–64.

Zhang, H., Squirrell, J.M., and White, J.G. (2008). RAB-11 permissively regulates spindle alignment by modulating metaphase microtubule dynamics in Caenorhabditis elegans early embryos. Mol. Biol. Cell *19*, 2553–2565.

Zhang, H., Wang, Y., Wong, J.J.L., Lim, K.-L., Liou, Y.-C., Wang, H., and Yu, F. (2014). Endocytic pathways downregulate the L1-type cell adhesion molecule neuroglian to promote dendrite pruning in Drosophila. Dev. Cell *30*, 463–478.

Zhang, Y., Song, M., Cui, Z.S., Li, C.Y., Xue, X.X., Yu, M., Lu, Y., Zhang, S.Y., Wang, E.H., and Wen, Y.Y. (2011). Down-regulation of TSG101 by small interfering RNA inhibits the proliferation of breast cancer cells through the MAPK/ERK signal pathway. Histol. Histopathol. *26*, 87–94.

Zhang, Y., Li, W., Chu, M., Chen, H., Yu, H., Fang, C., Sun, N., Wang, Q., Luo, T.,

Luo, K., et al. (2015b). The AAA ATPase Vps4 Plays Important Roles in Candida albicans Hyphal Formation and is Inhibited by DBeQ. Mycopathologia *181*, 1–11.

Zhao, M., Li, X.-D., and Chen, Z. (2010). CC2D1A, a DM14 and C2 domain protein, activates NF-kappaB through the canonical pathway. J. Biol. Chem. *285*, 24372–24380.

Zheng, L., Saunders, C.A., Sorensen, E.B., Waxmonsky, N.C., and Conner, S.D. (2013). Notch signaling from the endosome requires a conserved dileucine motif. Mol. Biol. Cell *24*, 297–307.

Zhu, J., Palliyil, S., Ran, C., and Kumar, J.P. (2017). *Drosophila* Pax6 promotes development of the entire eye-antennal disc, thereby ensuring proper adult head formation. Proc. Natl. Acad. Sci. *114*, 5846–5853.

Zhui, G., Gilchrist, R., Borley, N., Chng, H.W., Morgan, M., Marshall, J.F., Camplejohn, R.S., Muir, G.H., and Hart, I.R. (2004). Reduction of TSG101 protein has a negative impact on tumor cell growth. Int. J. Cancer *109*, 541–547.

Zivony-Elboum, Y., Westbroek, W., Kfir, N., Savitzki, D., Shoval, Y., Bloom, A., Rod, R., Khayat, M., Gross, B., Samri, W., et al. (2012). A founder mutation in Vps37A causes autosomal recessive complex hereditary spastic paraparesis. J. Med. Genet. *49*, 462–472.

10 SUPPLEMENTARY DATA

Table S1: List of genes identified in the *Vps25RNAi* primary screen

Suppressors	Enhancers
CG10083	ndl
CG10147	Rpn6
CG1021	Hip1
LanA	CG10973
mbc	dgrn
alphaKap4	CG11241
eyg	CG11255
Itp-r83A	CG11267
toe	Rcd5
Vps36	VhaM9.7-c
Chro	Nc73EF
Fur1	vps2
Madm	mei-P22
Sap130	Fit1
Ssl1	ImpL2
slif	Vha55
CG11253	bin
mael	disp
ste14	aralar1
Syx13	TER94
Mul1	aur
Abd-B	ClC-a
Ras64B	twin
CG11876	Jupiter
hyx	sals
CG11997	Pc
CG12163	VAChT
tipE	sav
pav	Not1
btz	CG3764
CG13025	Baldspot
mats	Abl
stg	Pcaf
tkv	Csk
Bub1	plx
CG14128	bnl
CDase	lack
Chd64	FBgn0011666
ens	Irbp
RfC4	ClC-c
mas	wkd

CG17360	sds22
CG17364	pll
Lnk	crb
CG18404	lsn
Wnt2	Sbf
Mad1	CG6962
sec8	Dic61B
Tina-1	AP-50
FBgn0016061	Rack1
CG31145	CG7146
Dscam3	CG7369
repo	hbs
wry	Mekk1
CG32113	Fit2
RhoGAP71E	mrn
l(3)76BDr	sim
SPoCk	ran-like
CG3339	sr
U3-55K	shrb
Rim	gro
CG33970	Vps16A
Rab26	CG8546
okr	wun
jumu	Shal
CG4098	Ras85D
l(3)73Ah	brv1
TFAM	CG9775
lqfR	pinta
Nedd4	CG7023
Gap69C	CG31100
cnc	mgr
scrib	VhaM8.9
Irk2	
XNP	
CG4553	
hh	
CG4849	
Syx7	
l(3)72Dh	
Pgm	
Pli	
Zn72D	
CG5235	
Taspase1	
loco	
Taf4	

cln3	
Prm	
Arr2	
cdi	
Pep	
sec10	
Apc2	
Rfx	
Dcr-2	
FBgn0001168	
CG6498	
AGO1	
oa2	
Saf-B	
Nrx-1	
Mkrn1	
htl	
CG7255	
byn	
AGO2	
gwl	
CG7804	
PP2A-B'	
CG7963	
Su(z)12	
Vps45	
Rab8	
ATPCL	
cg	
lqf	
sec63	
wun2	
Fps85D	
Gmd	
Hsc70-1	
by	
Dab	
Nrt	
pum	
CG32109	
Aos1	
CG33293	
CG17184	
trc	