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Characterization of the Mammalian homologs of the Drosophila Melanogaster Endocytic Protein Lethal (2) Giant Discs 1

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Résumé

Endocytose joue un rôle dans l'activation du récepteur Notch. Des mutations dans le gène drosophilien *lethal giant discs* (*lgd*), provoque une prolifération cellulaire en perturbant l'endocytose de Notch. Les orthologues murins mlgd1 et 2 peuvent sauver ce phénotype, démontrant une fonction conservée. Cependant, des publications récentes suggèrent que les orthologs humains de *lgd* (hgd1/2) sont nucléaires. Dans cette étude, il est démontré que chez la *Drosophile*, le mutant $dlgd^{08}$ provoque l'accumulation de Notch dans des vésicules et une surprolifération de neuroblastes . Ceci suggère que Notch est activé a l'intérieur des endosomes dans les neuroblastes. L'immunohistochimie de cellules Hela indique que hlgd1 et 2 ne sont pas nucléaires, mais associés à des strctures endosomales. Enfin, la baisse d'expression par shRNA des gènes murins mlgd1 et mlgd2 provoque une différenciation accélérée des cellules souches hématopoïétiques dans la lignée lymphopoïèse T et bloque la transition DN3 / $CD4^{+}CD8^{+}$, suggérant une suractivation de Notch.

Mots-clés : Endocytose, Notch, lethal giant discs (lgd), neuroblats, hématopoïèses, lymphopoïèse T

Abstract

Endocytosis plays a role in the activation of the Notch receptor. Mutations in the *Drosophila* gene lethal giant discs (lgd), causes cellular overgrowth by perturbing Notch endocytosis. This *Drosophila* phenotype is rescued by the murine orthologs mlgd1 and 2, indicating conserved function. However, recent publications suggest that the human orthologs (hlgd1/2) are nuclear. This study demonstrates that the $dlgd^{08}$mutant in *Drosophila* causes accumulation of Notch in vesicles and the overproliferation of neuroblasts. This suggests Notch is activated from within endosomes in neuroblasts. Immunohistochemistry of Hela cells indicates that hlgd1 is associated with early endosome while, hlgd2 with later endosome and lysosome, and not with the nucleus. Finally, down regulation of murine *mlgd1* and *mlgd2* by shRNA caused an accelerated differentiation of hematopoietic stem cell into the T lymphopoiesis lineage and blocked the DN3 to $CD4^{+}CD8^{+}$ transition, suggesting that Notch is overactivated in these cells.

Keywords : Endocytosis, Notch, lethal giant discs (lgd), neuroblasts, haematopoiesis, T lymphopoiesis

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List of abbreviations

µg	Micro gram
5-HT1A	Serotonin receptor
ACD	Asymmetric cell division
ADAM	A disintegrin and metalloprotease
AGL	Apoptosis-linked gene
AGM region	Aorta, gonads, and mesonephros region
Akt	serine/threonine protein kinase
AP-2	Adaptor-protein-2
aPKC	Atypical protein kinase C
ARH	Autosomal recessive hypercholesterolemia protein
Arp	Actin-Related Proteins
Ase	Asense
ATP	Adenosine triphosphate
BAR	Bin–Amphiphysin–Rvs domain
bHLH	Basic helix-loop-helix
C2 domain	Calcium-binding motif
Ca2+	Calcium
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
Cav	Caveolin
CBP	cAMP-response element-binding (CREB) binding protein
CC2D1	Coiled-coil and C2 domain containing 1
CC2D1A	Coil-coil containing domain
cDNA	complementary deoxyribonucleic acid
CLP	Common lymphoid progenitors
CME	Clathrin-mediated endocytosis
Cre	Cyclization recombination
CSL	CBF1/Drosophila Su(H)/C. elegans LAG-1
C-terminus	Carboxyl-terminus

DC	Dendritic cell
Dl	Delta-like
Dlg	Discs large
dlgd	Drosophila lethal giant discs
DN	Double-negative
DP	Double positive
Dpn	Deadpan
DRE	Dual repressor element
DSL	Delta, Serrate and Lag 2
dx	Deltex
E	embryonic day
E'(Spl)	Enhancer of Split
E. Coli	*Escherichia coli*
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
EE	Early endosome
EEA1	Early Endosome Antigen 1 protein
EFC/F-BAR	Extended FCH Homology / FCH- Bin–Amphiphysin–Rvs domain,
EGF	Epidermal growth factor
EHD	Eps15-homology domain-containing protein
ENTH	Epsin N-terminal homology domain
ept	Erupted
ESCRT	Endosomal sorting complex for transport
ETP	Early T-cell precursor
FACS	Fluorescence-activated cell sorting
Fbw	F-box and tryptophan-aspartic acid (WD) repeat domain-containing
Freud	Five repressor element under dual repression-binding
FYVE domain	Fab 1, YOTB, Vac 1 (vesicle transport protein), and EEA1 (Early Endosome Antigen 1 protein)
GAK	Cyclin G-associated kinase

GLUE domain	GRAM like ubiquitin binding in EAP45 domain
GMC	Ganglion mother cell
GSL	Glycosphingolipid
GST	glutathione S-transferase
GTP	Guanosine triphosphate
$G_{\alpha i}$	G protein subunit
HECT	Homologous to E6-associated protein carboxyl-terminus domain
Hes5	Hairy and enhancer of split 5
HIP1/R	Huntingtin-interacting protein 1-related protein
hlgd	Human lethal giant discs
HOPS	Homotypic fusion and vacuole protein sorting
Hprt	Hypoxanthine guanine phosphoribosyl transferase
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
HSC	Hematopoietic stem cell
Hsc70	Heat shock protein cognate of 70kDa
ILV	Intralumenal vesicles
Insc	Inscuteable
JAK	Janus kinase
Kda	kilodaltons
KO	Knockout
LAMP	Lysosomal-associated membrane protein
LBPA	Lysobisphosphatidic acid
LDL	Low-density lipoprotein
lgd	Lethal giant discs
Lgl	Lethal (2) giant larvae
LMPP	Lymphoid primed multipotent progenitors
LRF	Leukemia/lymphoma-Related Factor
LTR-HSC	Long-term repopulating hematopoietic stem cell
M/G	myeloid
Mam	Mastermind
Mb	Megabase

Mib	Mind bomb
Mira	Miranda
ml	Milli litter
mlgd	Murine lgd lethal giant discs
MOI	Multiplicity of infection
MPP	Multipotent progenitors
Mvb	Multi-vesicular body sorting factor
MVB	Multivesicular bodies
N1	Notch1
NB	Neuroblast
Neur	Neuralized
NEXT	Notch extracellular truncation
NICD	Notch intracellular domain
NK cell	Natural-killer cell
NSCL	Neuronal stem cell leukemia
NSMR	Non-syndromic mental retardation
N-terminus	Amino-terminus
Nuf	Nuclear fallout
nuroD	neurogenic differentiation
N-WASP	Neural Wiskott-Aldrich syndrome protein
PCAF	P300/CBP-associated factor
PDK	3-phosphoinositide-dependent protein kinase
PEST domain	Proline (P), glutamic acid (E), serine (S), and threonine (T)
PH domain	Pleckstrin homology domain
PI3K	Phosphoinositide 3-kinase
Pins	Partner of inscuteable
Pon	Partner of Numb
Pros	Prospero
PSAP	proline, serine, alanine, and proline amino acid motif
PtdIns-3-P	Phosphotidylinositol-3-phosphate
PtdIns-4,5-P_2	Phosphatidylinositol(4,5)bisphosphate

PX domain	Phosphoinositide-binding structural domain
Rab	Ras-associated binding
Rab11FIP	Rab11 family-interacting protein
RE	Recycling endosomes
RING	Really interesting new gene domain
RNAi	Ribonucleic acid interference
SCD	Spondylocostal dysostosis
SEL-10	Suppressor/Enhancer of Lin-12
Ser	Serine
SH3	Src homology 3 domain
shRNA	Small/short hairpin ribonucleic acid
SIN-LTR	Self inactivating long terminal repeats
siRNA	Small interfering ribonucleic acid
SJ1	Synaptojanin-1
SNARE proteins	Soluble NSF attachment protein receptors
SNX	Sorting nexin
SOP	Organs precursor cells
SP	Signal positive
STAM	Signal transducing adaptor molecule
STAT	Signal transducers and activators of transcription
Su(H)	Suppressor of Hairless
SV40	Simian virus 40
SVZ	Subventricular zone
T-ALL	Cell Acute Lymphoblastic Leukemia
Tbp	TATA box binding protein
TCR	Pre-T-cell receptor
Thr	Tyrosine
Tsg101	Tumour susceptibility gene 101
TSP	Thymus-seeding progenitor
TTK69	Tramtrack p69
Ub	Ubiquitin

UEV domains	Ubiquitin-conjugating E2 variant domain
UIM	Ubiquitin interacting motif
Vps	Vacuolar protein sorting factor
VZ	Ventricular zone
WT	Wild-type
WW domain	Proline-rich peptide motifs
WWC	WW domain-containing protein

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Introduction

1.1. Rationale of the current study

Endocytosis is the process where cell surface receptors and soluble molecules present at the plasma membrane or in the extracellular medium are internalized into intracellular compartments. It has been demonstrated that endocytosis can regulate numerous processes, including: nutrient uptake, receptor signalling[1], cell adhesion and migration[2], cell polarity[3], pathogen entry[4], antigen presentation[5], neurotransmission[6], mitosis[7], growth and differentiation[8], and drug delivery. The Notch signalling cascade has been shown to be regulated by endocytosis. The transmembrane Notch receptor has been demonstrated involved in cell fate specification in every animal species studied so far[9]. Notch signalling is essential to many developmental processes; this includes haematopoiesis[10], neurogenesis[11], and vasculogenesis[12] in mammals. It also regulates stem cell self-renewal, cell proliferation, cell differentiation and apoptosis. Loss of function of various components of the Notch signalling pathway causes inherited genetic diseases such as Alagille syndrome, spondylocostal dysostosis (SCD), and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)[13]. Notch is also a known oncogene and tumour suppressor in mammals[14, 15]. In human, mutations in one of the Notch homolog (NOTCH1) are involved in 50% of T-Cell Acute Lymphoblastic Leukemia (T-ALL)[16]. Levels of the different Notch isoforms have been shown to be increased in almost all T-ALL. Growth arrest occurs in several T-ALL derived cell lines when Notch signalling is blocked, suggesting that modulating Notch activity may be a potent treatment strategy for some cancers[17].

Notch signalling is regulated at numerous levels. The Notch receptor itself undergoes a series of modification to insure proper response to the different ligands (Delta and Serrate in Drosophila and Delta-like and Jagged in mammals) and activation[18]. The expression and activities of the ligands are also tightly regulated. It has been demonstrated that endocytosis is necessary both in the signal-receiving cell and in the signal sending cell. Endocytosis of the ligands seems to produce a force on Notch that allows its photolytic cleavage by an ADAM-like proteases at the plasma membrane[19, 20]. This leads to another cleavage by presenilin which occurs most-likely in endosomes[21, 22].

Furthermore, Notch has been shown to be downregulated in some cells by Numb, a protein that recruits the AP-2 adaptor complex, which in turn recruits clathrin and causes endocytosis of Notch[23, 24]. In some Notch expressing tissues, the absence of Numb can induce an accumulation of the receptor at the cell surface and an overactivation of Notch. This can lead to an overproliferation of cells and a tumour-like growth of tissues[25, 26]. Surprisingly, accumulation of Notch in endocytic compartments can also cause a Notch overactivation and overgrowth of proliferating tissues. For example, this is the case in *Drosophila* when the endocytic proteins vps25 or erupted (ept) are missing[27, 28]. Interestingly, ept is the homolog of the tumour susceptibility gene 101 (tsg101) that has been shown to be implicated in numerous types of human cancer[29-33]. Finally, in some circumstances, Notch can also be activated through a ligand-independent mechanism. Accumulation of Notch in endosomes could favour this unusual mechanism[34-36].

The Drosophila gene lethal giant discs (lgd), named after its loss of function phenotype has been shown to play a role in the regulation of the Notch signalling. Mutations in this gene cause overgrowth of the imaginal discs through a perturbation in Notch endocytosis[37-40]. The mammalian homologs of lgd are named CC2D1A and B or Freud-1 and -2 or mLgd1 and 2. For homogeneity, the Drosophila Lgd will be named dLgd, the mouse homologs respectively mLgd1 and 2 and the human homologs hLgd1 and 2.

Though dLgd has been characterized to function in the endocytic pathway, its precise function is unknown. Even less is known of the mammalian lgd1 and 2. In a Drosophila loss-of-function experiment, both *mlgd1* and *2* were able to rescue the *dlgd* loss-of-function phenotype, thus demonstrating a conservation of function between homologs[39]. However, recent publications suggest that they may act differently in mammals than in *Drosophila*[41-44]. In mammals, published data shows or strongly suggest that the Lgd homologs are not endocytic proteins but nuclear[41, 43, 44].

The aim of this study was to determine the role of the *Drosophila* and mammalian Lgd in the endocytic pathway and to verify the conservation of its function in mammals.

The literature review below provides a brief overview on the various forms of endocytosis and the role of endocytosis on Notch signalling. The role of Notch signalling in the hematopoietic system, brain and cancer development will also be looked at. A synopsis on what is known on *Drosophila* and mammalian Lgd will conclude this section.

1.2. Endocytosis

The plasma membrane is a dynamic structure that segregates the intracellular milieu (the cytoplasm) from the extracellular environment. It regulates and coordinates the entry and exit of small and large molecules from the cell. Small molecules, such as amino acids, sugars and ions, can pass through the plasma membrane via passive diffusion or through the action of integral membrane protein pumps or channels. The process of invagination and pinching-off of pieces of the plasma membrane, known as endocytosis, is needed to internalize macromolecules in membrane bound vesicles into the cell. There are multiple forms of endocytosis which can be place into two broad categories, 'phagocytosis' or cell eating (the uptake of large particles) and 'pinocytosis' or cell drinking (the uptake of fluid and solutes)[45]. Phagocytosis usually occurs in specialized mammalian cells such as macrophages, monocytes and neutrophils that function to clear large pathogens like bacteria, yeast, or large debris such as the remnants of dead cells and arterial deposits of fat[46, 47]. Pinocytosis occurs in all cells by at least one of four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME), caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis[45]. These mechanistically diverse and highly regulated endocytic pathways function to control such complex physiological processes as nutrient uptake, receptor signalling[1], cell adhesion and migration[2], cell polarity[3], pathogen entry[4], antigen presentation[5], neurotransmission[6], mitosis[7], growth and differentiation[8], and drug delivery.

1.2.1. Clathrin-Mediated Endocytosis

Clathrin-dependent endocytosis is the major pathway for the uptake of nutrients and signalling molecules in higher eukaryotic cells. The formation of endocytic-coated vesicles start by the recruitment of clathrin, adaptors and endocytic accessory proteins to the plasma membrane where they form lipid-rafts ranging from 10 to 500 nm in diameter[48]. Proteins to be endocytosed are linked to the plasma membrane by coat proteins that bind both phosphatidylinositol(4,5)bisphosphate (PtdIns-4,5-P_2), which is the prevalent phosphoinositide species of the plasma membrane, and transmembrane cargo[49]. There

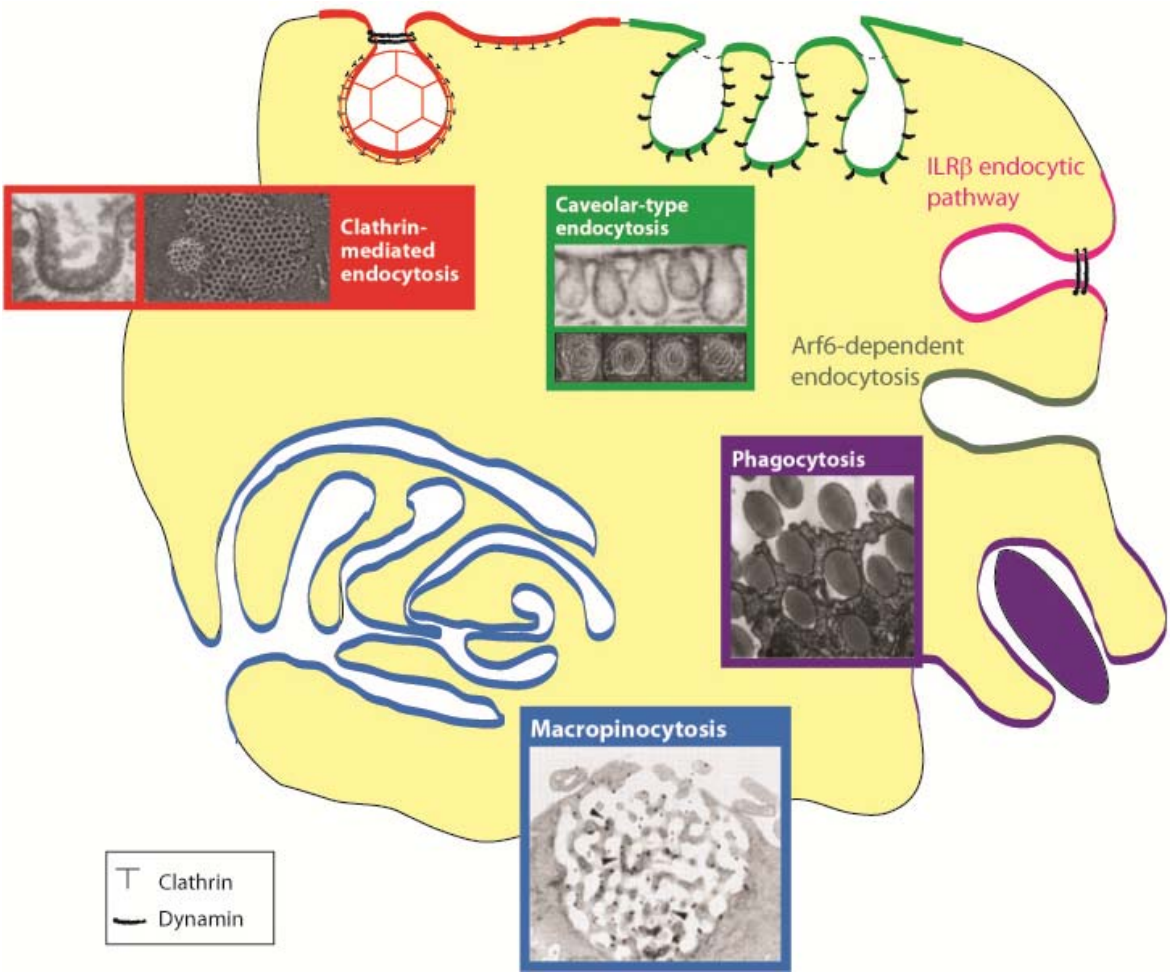


Figure 1: Different types of endocytosis. Transmission and scanning electron micrographs, and fluorescence micrographs, of structures known or thought to be involved in endocytic events. This figure was modified from *Doherty and McMahon (2009)*[50]. Note that there is controversy surrounding the morphologies of the IL2Rβ pathway and Arf6-dependent pathway. The arrowheads in the macropinocytic picture indicate cytoskeletal elements.

are four types of internalization signals identified located in the cytoplasmic tails of transmembrane proteins that are recognized by the endocytic adaptor proteins: the YXXΦ-, the [DE]XXXL[LI]-, the FXNPXY-type internalization signals and polyubiquitination. These signals are recognized by two types of endocytic adaptor proteins: the tetrameric adaptor-protein-2 (AP-2) and the monomeric adaptors ARH (autosomal recessive hypercholesterolemia protein), Dab2, β-arrestin, numb and epsin[51, 52]. Most adaptors are able to bind clathrin and the monomeric adaptors can also associate with AP-2. These adaptors are targeted to the plasma membrane through a phosphoinositide binding modules that associate preferentially with PtdIns-4,5-P_2[49]. The AP-2 adaptor plays a central role since it can associate with most endocytic proteins[53]. The loss-of-function of AP-2 subunits was proven to be lethal in *Drosophila*, *C. Elegans* and mice[54-56]. Also siRNA or RNAi mediated knockdown of AP-2 expression in HeLa cells eliminates about 90% of the endocytic clathrin-coated structures and blocks transferring uptake[57, 58]. However, even in the absence of AP-2 the Dab2 and epsin adaptors continue to mediate the uptake of LDL[58] and ubiquitinated cargo[59], respectively. These observations suggest that clathrin-mediated endocytosis can occur without AP-2.

There are several ways of bending a membrane which includes the enrichment of cone-shaped lipids in the cytoplasmic leaflet of the membrane, insertion of protein into the cytoplasmic leaflet, binding of coat proteins with intrinsic curvature, and force exerted by the cytoskeleton[60]. The presence of clathrin and AP-2 was demonstrated to be essential for the invagination of coated structures, however it is not sufficient[61]. Members of the ENTH, BAR and EFC/F-BAR protein families have been shown to induce, sense and stabilize membrane curvature. For example, upon binding to PtdIns-4,5-P_2 Epsin inserts a short amphipathic helix into the cytoplasmic leaflet of the plasma membrane thus bending the bilayer. Transient over-expression of the epsin ENTH domains cause tubular membrane structures in cultured cells[62]. However, when clathrin is depleted in HeLa cells, the presence of epsin and AP-2 is not sufficient to induce curvature[61]. This may be due to a requirement of clathrin for epsin to be able to curve the membrane or epsin may not be present in sufficient concentrations to bend the plasma membrane in the clathrin-deficient Hela cells. Depletion by RNAi of epsin in HeLa cells does not impede clathrin-dependent endocytosis of transferring but interfere with the uptake of ubiquitinated cargo[59].

It is thought that the clathrin lattice induces, directly or indirectly, changes in the curvature of coated membrane patches by its inherent property to form a curved polyhedral network. It is theorized that synergistic contributions obtained from interactions between adaptors and clathrin, and from proteins within the cytosolic membrane leaflet are required to effective bend the membrane. The elastic spring-like linkage between the clathrin lattice and the membrane might allow for local changes in the curvature of either the membrane or the clathrin lattice without too much resistance by the respective other. In order to convert the hexagonal facets of clathrin into pentagonal ones clathrin needs to associate with local promoter to increase the lattice curvature. There is evidence that the uncoating ATPase Hsc70 and its co-chaperone GAK/auxilin7 may be involved in this process. It has been demonstrated that low levels of GAK/auxilin [63] and of the 170 Kda isoform of synaptojanin-1 (SJ1), a phosphatidylinositol phosphatise, are recruited together with clathrin in growing coated patches at the plasma membrane[64].

The first event leading to scission and the release of a coated vesicle into the cytoplasm is the recruitment of dynamin GTPase. This most-likely occurs through curvature-sensing proteins[63, 65, 66]. Then cortactin, N-WASP, Arp2/3, actin, endophilin and the 145 kD isoform of Sj1 are recruited[64, 67]. GAK/auxilin is recruited in a short burst afterward[63]. The accessory protein HIP1/R, which is recruited together with clathrin into growing coated structures, connects the clathrin coat to actin filaments[68].

There are two theories on how these actin filaments help in scission of the coated vesicle into the cytoplasm. 1) The formation of actin filaments at the constricted neck of the budding vesicle may push the bud deeper into the cytoplasm and the increasing strain on the stalk may cause it to sever[49]. 2) Recent findings implicate the involvement of actin motor proteins in endocytosis. Myosin VI was shown to attach to coated vesicles through to the accessory protein Dab2 and PtdIns-4,5-P_2 [69] and myosin 1E to dynamin and SJ1 through its SH3 domain[70]. So it is thought that the plus-end motor myosin 1E may pull the dynamin ring toward the plasma membrane while the minus-end motor myosin VI pulls the vesicle into the cytoplasm. The strain produced from these opposing forces would sever the constricted stalk beneath the dynamin ring[49].

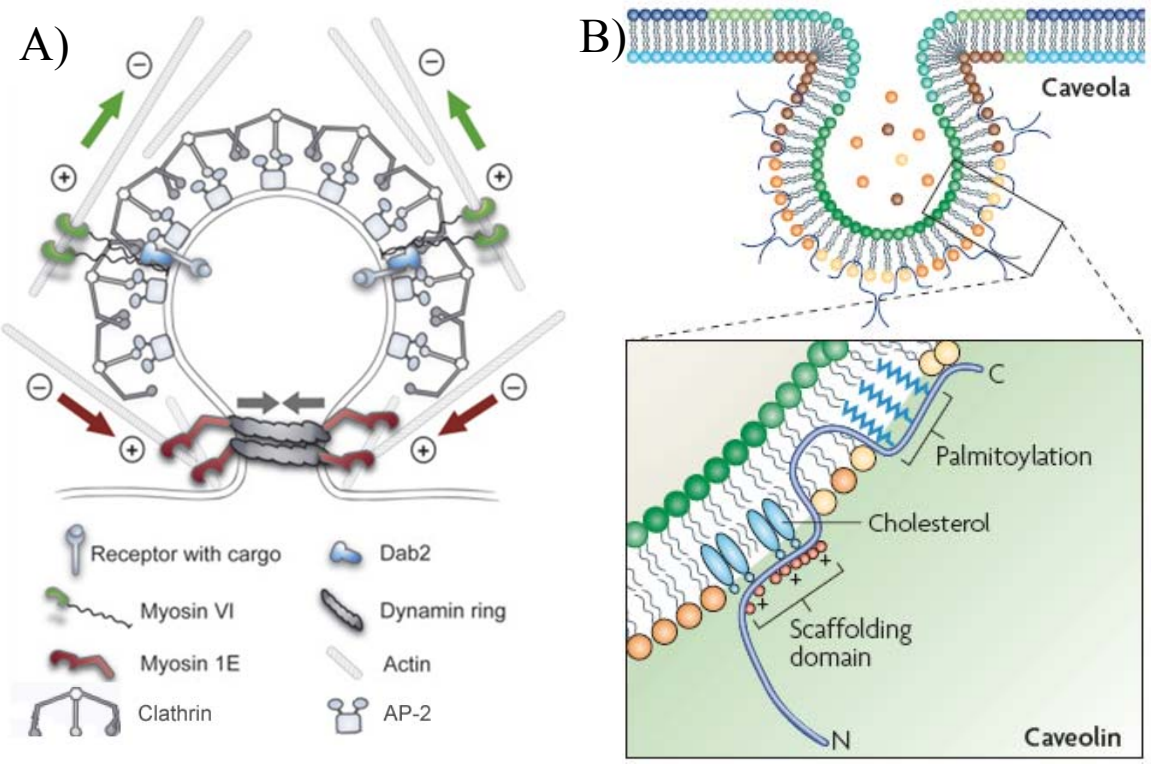


Figure 2: Clathrin (A) and Caveolin (B) mediated vesicles. A) Diagram of the assembly and abscission of clathrin coated pits taken from *Ungewickell & Hinrichsen, 2007*[49]. The adaptor proteins, in this case AP-2, interact with both cargo protein and the cathrin lattice. Both the insertion of certain adaptor proteins into the membrane and the clathrin lattice help bends the membrane into the cell. Constriction of the vesicle neck by dynamin and additional pulling force provided by myosin motor proteins allow for vesicle fusion. B) Diagram of a caveola taken from *Parton et al., 2007*[71]. It demonstrates how caveolin is inserted into the caveolar membrane, with the N and C termini facing the cytoplasm and a putative 'hairpin' intramembrane domain embedded within the membrane bilayer. The scaffolding domain, a highly conserved region of caveolin, might have a role in cholesterol interactions through conserved basic (+) and bulky hydrophobic residues (red circles). The C-terminal domain, which is close to the intramembrane domain, is modified by palmitoyl groups that insert into the lipid bilayer.

1.2.2. Ubiquitin-Regulated endocytosis

Ubiquitination is a reversible, post-translational modification that is the result of the conjugation of small 76 amino acids ubiquitin (Ub) protein to a lysine residue on a target protein.[72, 73] Ubiquitin itself can be ubiquitinated on one of its 7 lysines and polymerize into either short chains (oligoubiquitination) or longer chains (polyubiquitination). The length and the topology of these chains determine the fate of the ubiquitylated protein. Modification of a protein by ubiquitin (Ub) can cause a remodelling of the targeted protein by affecting their stability, interaction with other proteins, enzymatic activity, and their subcellular localization[72, 73].

Successive action of the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3) is required for ubiquitylation of proteins. Based on structural features, E3s can be classified into two categories: 1) the E3s with a RING domain (Really Interesting New Gene), which act as molecular scaffolds to bridge the E2 and substrates together to allow the transfer of ubiquitin, and 2) the E3s with a HECT domain (Homologous to E6-AP C-Terminus), where the ubiquitin moiety is first covalently attaches to the E3 before it is transferred onto the substrate[72, 73]. Both E3s dictating the specificity of the ubiquitylation reaction since they usually interact directly with the substrate.

The Nedd4/Rsp5p family of ubiquitin-protein ligases regulate the stability of several yeast and mammalian transmembrane proteins by ubiquitination, and targets them for subsequent endocytosis. The Nedd4/Rsp5p family belongs to the Hect-domain superfamily of E3 enzymes. They are all composed of a variable N terminus, a C2 domain, 2 to 4 WW domains and a C-terminal Hect domain[74]. The C2 domain binds phospholipids and membranes in a Ca2+-dependent manner and plays a role in membrane targeting, intracellular localization and trafficking of proteins[75]. The WW (or WWP) domain is a small (~40 aa) protein-protein interaction module that allows Nedd4/Rsp5 to form multiple interactions and complexes with various proteins simultaneously. It usually recognizes and ubiquinates proteins that contains a conserved PPxY motif.

Once targeted plasma membrane proteins are ubiquitinated, they are recognized by Ub binding domains (UBDs) containing proteins such as the endocytic adaptors eps15 or epsin[76]. The ubiquitinated proteins are then endocytosed in a clathrin-dependent manner.

1.2.3 Caveolae-dependent endocytosis

Caveolae, which are flask-shaped invaginations of around 60–80 nm in diameter, are found in many mammalian cells including in smooth muscle, type I pneumocytes, fibroblasts, adipocytes, and endothelial cells[71]. Caveolin-1 (Cav1) and Cav2 are abundant in non-muscle cells, while Cav3 is found in skeletal muscle and in some smooth-muscle cells[77]. Transient expression of Cav1 is sufficient for de novo formation of caveolae in cells that do not express caveolin[78]. Ablation of CAV1 or CAV3 causes loss of caveolae formation in their respective cell types[79, 80]. Loss of CAV2 has no effect on caveola formation in vivo however there is some evidence that it may contribute to caveola formation in certain cell types[81]. All three caveolins have an unusual topology. They have a 33-amino acid intramembrane that forms a hairpin loop that inserts into the plasma membrane which leaves the N and C terminals in the cytoplasm[82]. Caveola formation by Cav1 and Cav3 involves oligomerization and association with cholesterol-rich lipid-raft domains. Cav1 binds to 1–2 cholesterol molecules[83] and is also palmitoylated in the C-terminal region[84]. The depletion of cholesterol has been demonstrated to disrupt caveolae structure[85]. Cav1 has been shown to bind the fatty acid tails of the glycosphingolipid (GSL) GM1 and can colocalize with GM1 and another “raft-associated” GSL, Gb3, in cellular membranes[86]. Caveolae endocytosis is dependent on dynamin. In endothelial cells dynamin is constitutively localized to the neck of caveolae[87], or is recruited in response to specific signals, such as stimulation by the SV40 virus[88]. The SV40 virus enters the cell partially through caveolae. It binds to GM1 and causes a transient recruitment of dynamin, which is followed by a burst of actin polymerization. The actin forms a tail that pushes the caveolae into the cell[88]. This sequence of events is similar to that observed in clathrin endocytosis.

Phosphorylation appears to play an important part in caveolae budding. It has been shown that the use of the general phosphatase inhibitor okadaic acid and the tyrosine

phosphatase inhibitor vanadate can stimulate caveolae internalization[88, 89]. However in contrast, the Src family of kinases has been demonstrated to be able to phosphorylate both Cav1 and dynamin and that phosporylation of dynamin is directly involved in caveolae internalization[88, 90]. Thus, the precise role of these phosphorylations is presently unclear.

1.2.4. The Early endosome

The early endosome (EE) is the first endocytic compartment to receive incoming internalized cargo from the plasma membrane. It is a highly dynamic structure that can undergo homotypic fusion[91]. The EE is composed of two distinct regions: 1) thin tubular extensions and 2) large vesicles that have membrane invaginations. These EE sub-domains appear to have different function. It is thought that proteins targeted for recycling may cluster within the tubular membranes, while proteins destined for degradation concentrates on intralumenal vesicles that accumulate within the vacuolar domains, giving rise to multivesicular bodies (MVBs)[92]. Vesicles generated from these two regions have different acidification properties. In the lumen of MVB, the pH decreases from 6.2 to ~5.5, while in the tubular recycling endosomes it increases to ~6.5[92].

The Ras-associated binding (Rab) proteins are GTP-binding proteins that are important endocytic regulators. In their active GTP-bound state, they can recruit and interact with Rab effectors. The Rab proteins have multiple roles in endocytic trafficking events, including vesicle tethering, fusion, budding and motility[93, 94]. The Rab proteins primarily localized to the EE include Rab5 and Rab4, which regulate distinct early endocytic events. Rab5's main role is to regulate entry of the cargo from the plasma membrane and subsequent fusion of vesicles with early endosomes[95, 96]. Rab5 has multiple secondary functions as well, which include: generation of phosphotidylinositol-3-phosphate (PtdIns-3-P) lipid which is enriched on EE[97], homotypic fusion[98], the motility of EE on actin and microtubules tracks[99], and functions in activating signalling pathways from EE[100]. Rab4 is localized in the EE[101] and in Rab11-positive recycling endosomes (RE)[102] as well. It helps regulates both rapid recycling of proteins from the EE and the slow recycling of proteins from the RE back to the cell surface[103].

Phospholipids play a role in recruiting proteins to specific domains of the EE. Active Rab5 recruits PtdIns-3-P kinase/Vps34 to EE membranes where it mediates localized synthesis of PtdIns-3-P[97]. This localized accumulation of PtdIns-3-P can recruit PtdIns-3-P-binding proteins containing a FYVE domain, such as EEA1 or Hrs, and containing PX domain, like Syntaxin 1[104, 105]. The recruitment of EEA1 can cause the homotypic fusion between EE through the assembly of SNAREs Syntaxin13 and Syntaxin6 at the EE membranes[106]. While the recruitment of Hrs is essential for the sorting of ubiquitinated proteins into MVBs, which are then targeted for lysosomal degradation.

1.2.5. The recycling endosome

Upon delivery to EE, internalized cargo can be sorted into one of at least two distinct recycling pathways. Proteins sorted into the newly formed tubular membranes of the EE are recycled back directly to the plasma membrane. In parallel with tubulation, Rab4 targets proteins to the recycling endosome (RE) where they proceed via a 'slow-recycling' route back to the plasma membrane[107].

Rab4 has been identified to also be involved in the fast recycling of transferrin receptor[108] and glycosphingolipids[109] from the EE. However its exact role in recycling is unclear for expression of dominant-negative Rab4 inhibits fast recycling but not slow recycling and siRNA-mediated down-regulation of Rab4 increases rapid recycling[107, 108]. Recent studies have indicated that Rab35 is an important regulator of rapid recycling. Rab35 has been shown to localize both at the plasma membrane and EE, and to be required for rapid recycling of the mammalian transferrin receptor[110] and the *C. elegans* low-density lipoprotein receptor-like yolk receptor[111].

The slow recycling route involves the transport of cargo proteins from the EE to the RE and from the RE to the plasma membrane. The EH domain-containing protein (EHD) proteins appear to play many roles in the RE. EHD4 is involved in the export of cargo from the early endosome to both the RE and late endosome[112], while EHD3 act as linker between the Rab5-associated early endosome and the Rab11-associated RE. It can bind to the Asn–Pro–Phe motifs of the Rab5 effectors rabenosyn and Rab11 family-interacting protein 2 (Rab11FIP2)[113]. Rab11FIP5, another Rab11 effector, is important in the

movement of transferrin receptor from the EE to the RE. SiRNA down-regulation of Rab11FIP5 inhibits the transport of transferrin receptor from the early endosome to the RE[114]. Rab11FIP3 interacts with both Rab11 and Arf6 and is important for the juxtanuclear positioning of the RE[115].

The sorting nexins protein in yeast, and its mammalian ortholog, sorting nexin 4 (SNX4), associate with tubular vesicular elements on the both EE and RE, and target cargo for the recycling pathway. SNX4 interacts with the dynein motor through the linker protein WW domain-containing protein 1 (WWC1), facilitating the movement of the EE or RE to the juxtanuclear region[116]. Down-regulation of SNX4 perturbs transport between these compartments and causes lysosomal degradation of the transferrin receptor[116].

1.2.6. The ESCRT complex and sorting for the lysosomal degradation

Mono-ubiquitination of one or more lysine residues of endocytosed cargo serves as recruiting signal for the endosomal sorting complex for transport (ESCRT) machinery[117]. The ESCRT machinery has three main functions: 1) it recognizes ubiquitylated cargoes and prevents their recycling and/or retrograde trafficking; 2) it deforms the endosomal membrane allowing cargo to be sorted into endosomal invaginations; 3) it catalyses the final abscission (breaking off) of the endosomal invaginations, forming intralumenal vesicles (ILVs) that contain the sorted cargo[118]. The hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a member of the ESCRT-0 complex, is first recruited to the EE through binding of its FYVE zinc-finger domain to PtdIns-3-P[105]. Mono-ubiquitinated cargo on the EE is initially recognized by Hrs via its ubiquitin interacting motif (UIM)[105]. Hrs can also associates with clathrin through its C-terminal clathrin box motif. Hrs has been found on clathrin lattices present on select EE membranes involved in the sorting of cargo to the degradation pathway[118]. Binding of Hrs to ubiquitin and clathrin results in the concentration and sorting of cargo into ILVs vesicles, thus away from the tubule-forming and actively recycling membranes. However, Hrs binds to ubiquitin with a low affinity[105, 119], thus two additional UIM-

containing proteins make up the ESCRT-0 complex. Eps15 and signal transducing adaptor molecule (STAM) 1 and 2, stabilize the association of ubiquitinated cargo with Hrs[120]. Furthermore, Hrs interacts with the Tsg101 subunit of the endosomal sorting complex required for transport (ESCRT-I), thus recruiting it to membranes[120].

ESCRT-I is composed of four subunits: tumour susceptibility gene (Tsg) 101, vacuolar protein sorting (Vps) 28, Vps37 and multi-vesicular body sorting factor (Mvb)12[121, 122]. The ESCRT-I complex is recruited to the endosome through the interaction of the amino acid motif proline, serine, alanine, and proline (PSAP) of Hrs with the ubiquitin-conjugating E2 variant (UEV) domains of Tsg101[123]. It is believed that the ubiquitinated cargo is then handed off from Hrs to Tsg101, which also contains a UIM[123]. ESCRT-II is recruited by Vps28, which is located at the end of a rigid 13-nm stalk opposite to the binding sites for ubiquitinated cargo[124]. With such a distance between the ubiquinated cargo and the ESCRT-II complex, it argues against the 'conveyor belt' concept. This concept was first suggested in a study by *Babst, M. et al, 2002* and supported by subsequent studies on ESCRT proteins. This model states that the recruitment of the ESCRT complexes occurs sequentially to the endosome. The ESCRT complexes recognize ubiquitylated transmembrane proteins and pass the ubiquinated cargo from one complex to the next facilitating sorting to MVB vesicles[125]. It is thought that ESCRT-I and ESCRT-II may co-assemble and cluster multiple ubiquitinated proteins for packaging into ILVs[124]. ESCRT-II is composed of Vps22, Vps36 and Vps25. The c-terminal domain of Vps28 binds to the N-terminus of the Vps36. The N-termini of Vps36 also contains a pleckstrin homology (PH) domain variant called a 'GLUE' domain. This GLUE domain binds to both phosphoinositides and ubiquitin[126]. There are six ESCRT-III core proteins in yeast, Vps2, Vps20, Vps24, Snf7, Did2, and Vps60. Vps20 is first recruited to the endosome and activated by Vps25[127]. It forms dimers with Snf7 and associates with Vps32. This association with Vps20 triggers the assembly of Vps32 into filamentous oligomers that are capped by Vps24[128]. Vps2 then associates with the Vps24 cap to mediate recruitment of the ATPase Vps4[128]. The ESCRT-III subcomplex of Did2 and Vps60 recruitments and regulates the activity of Vps4[129].

There is some evidence of ESCRT-mediated membrane deformation obtained with ESCRT-III. Overexpression of the ESCRT-III subunit Vps32 in COS-7 cells caused the

formation of spiral filaments composed of Vps32 multimers, which lead to the protrusion of buds and tubules from the plasma membrane[130]. The topology and diameter of the buds and tubules on the plasma membrane resemble those of ILVs, suggesting that Vps32-mediated plasma-membrane budding reflects key aspects of ILV biogenesis[130]. They are several lines of evidences suggesting that Tsg101 and one of its interacting partners, Alix (apoptosis-linked gene-2 (AGL-2) interacting protein X), are also involved in membrane deformation and intralumenal vesicles budding. The proline-rich domain located at the c-terminal of Alix interacts with Tsg101. Addition of purified recombinant Alix inhibits formation of intralumenal vesicles in the late endosomes in a dose-dependent manner while siRNAs depletion of Alix stimulated formation of intralumenal vesicles[131]. The reverse was observed when Tsg101 was knocked down by siRNA[131]. Alix was also found to interact with the phospholipid lysobisphosphatidic acid (LBPA), which is commonly found within the internal membranes of late endosomes. It was demonstrated that Alix controls LBPA's ability to drive the formation of membrane invaginations within acidic liposomes in vitro and helps organize LBPA-containing endosomes in vivo[132]. Recent studies done in Hela cells have identified the PtdIns-3-P-binding protein SNX3 as to be involved in ILV biogenesis. Depletion of SNX3 by RNAi prevents ILV biogenesis but not receptor sorting, indicating that it has a specific role in membrane deformation[133].

Abscission is thought to be mediated through the ATP hydrolyse activity of the ESCRT-III complex protein Vps4. The ESCRT-III subunits assemble into circular arrays on the LE. It is thought that Vps4-mediated removal of individual Vps32 subunits from one end of the spiral polymer at the neck of the invagination could cause sufficient constriction to mediate membrane scission[134]. It is also possible that ESCRT-III mediated clustering of cargo with bulky intraluminal domains could also contribute to both membrane deformation and abscission. Released MVB are transported and fused to the lysosome in a rab-7 dependent manner[135].

1.3. Notch signalling

The transmembrane Notch receptor was named in the early 1900s after a dominant X-linked *Drosophila* genetic mutants that exhibit irregular notches at the wing tips [136,

137]. However, it wasn't until the 1940s that Notch was shown to have a developmental role when complete loss of Notch gene activity was found to cause lethal hyperplasia of the embryonic nervous system[138]. Today it has been demonstrated that Notch signalling is essential to many developmental processes; this includes hematopoiesis[10], neurogenesis[11], and vasculogenesis[12] in mammals. It is involved in cell fate specification in every animal species studied so far[9]. Notch also regulates stem cell self-renewal, cell proliferation, cell differentiation and apoptosis. Loss of function of various components of the Notch signalling pathway causes inherited genetic diseases such as Alagille syndrome, spondylocostal dysostosis (SCD), and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL)[13]. Notch is also a known oncogene and tumour suppressor in mammals[14, 15]. Notch signalling can be either oncogenic or antiproliferative, in different types of cancers. In human hepatocellular carcinoma[139] and small cell lung cancer[140], Notch signalling is antiproliferative rather than oncogenic. However, studies have shown that Notch mostly functions as oncogene in human cancers. The expression of the Notch receptor and its ligans are up-regulated in cervical, lung, colon, head and neck, renal carcinoma, acute myeloid, Hodgkin and large-cell lymphomas and pancreatic cancer (as reviewed in [141]). Moreover, high-level expression of Notch-1 and its ligand Jagged-1 is associated with poor prognosis in breast[142] and prostate cancer.

Notch signalling regulates cell fate decision (or differentiation) by three general mechanisms during both development and adult life:

1) Inductive signalling occurs at the interface between two fields of cells, one of which is the signal-sending cell that presents the DSL family (Delta, Serrate and Lag 2) of Notch ligands to the signal-receiving cell that express the Notch receptor in neighbouring tissue. This type of interaction results in the formation of tissue boundaries, as is the case during fly wing and vertebrate limb development[143]. The dorsal/ventral boundary of the wing disc of drosophilas is formed by a stripe a few cell rows wide. In the dorsal cell, the Notch ligand Serrate is expressed and Notch is rendered insensitive to Serrate so that Serrate cannot activate Notch in these cells[144, 145]. Thus only the Notch receptor in the ventral cells across the boundary can be activated by Serrate. The reverse is true for the Notch

Delta ligand. Delta is expressed in the ventral cells, but can only activate Notch in the dorsal cells across the boundary[144, 145].

2) Lateral inhibition is the selection of one cell from a group of equivalent precursors. Initially all cells express both ligands and Notch receptor. However, as development progresses stochastic variations in gene expression cause small differences in Notch activation in the various cells so that in the end the ligand is restricted to a single cell while Notch is activated in its neighbours[143]. This can be seen in the equal spacing of sensory organs in the fly's sensory bristles on the thorax and with the hair cells in the ear of vertebrates[146, 147].

3) Asymmetric division occurs when a mother cell gives rise to two daughter cells with different fates. In some cases the two daughters are initially identical at birth and undergo the acquisition of different cell fate later on. Alternatively, the mother cell can become polarized and the two arising daughters receive different cell fate determinates at birth[148]. Stem cell epistatisi is thought to be regulated through this mechanism. When stem cells divide, one of the daughter cells retains stem cell status while the other daughter cell obtains cell fate determinates and differentiates. The dividing sensory organs precursor cells (SOP) is an excellent model system for asymmetric cell division, since the two resulting daughter cells receive different cell fate determinates[148]. The asymmetric division of SOPs yield two secondary precursor cells, the anterior cell, pIIb, and the posterior cell, pIIa. The pIIb receives cell fate determinants that activate the Delta ligand and down-regulate the Notch receptor, while the pIIa maintain an active Notch receptor at the plasma membrane[148, 149]. Activation of Notch signalling in the pIIa will gives rise to a socket cell and a hair cell, whereas the lack of Notch signalling in the pIIb will give rise to a neuron and a sheath cell[148, 149].

1.3.1. The role of endocytosis in Notch signalling

The Notch receptor is synthesized as a 300 kDa precursor protein which is cleaved in the trans-Golgi compartment by furin in *Drosophila* and furin-like convertases in mammals. Cleavage occurs in the extracellular/lumenal domain which results in the generation of N- and C-terminal fragments. These fragments are joined by a non-covalent

linkage and create the mature Notch heterodimer[150, 151]. The extracellular cellular domain of Notch contains numerous potential sites for N-linked and O-linked glycosylation and does undergoes extensive N- and O-linked glycosylation during Notch synthesis and secretion[152, 153]. These modifications are important for proper folding of the receptor and alter the responsiveness to the different DSL ligands[152, 153], which includes Delta and Serrate in *Drosophila*, and Delta-like and Jagged in mammals.

The canonical Notch signalling pathway (Figure 3.B.) involves activation of the Notch receptor at the cell surface by ligands of the DSL family. Both the Notch receptor and DSL ligands are composed mostly of type I single-pass integral membrane proteins with extracellular domains composed of tandem EGF-like repeats[154, 155]. The N-terminal domain of the ligand binds directly to the EGF-like repeat 11-12 region of the Notch receptor[156]. The ligand on the surface of a signal-sending cell must be internalized to activate the Notch receptor on the signal-receiving cell[157]. The DSL ligands are endocytosed through recognition of a ubiquitination signal. Neuralized (Neur) and Mind bomb (Mib) are two RING finger-containing E3 ubiquitin ligases that can ubquitinate the DSL ligans[158, 159]. This promotes the recruitment of liquid facet in *Drosophila*, Epsins in mammal, which contain a ubiquitin-binding domain. Espsin then binds the PtdIns-4,5-P_2 and associates with Clathrin and other accessory proteins[160] to induce clathrin-mediated endocytosis of the DSL ligans.

It is thought that DSL ligands may undergo constitutive endocytosis and recycling to and from the plasma membrane to produce active ligands[159]. It has been demonstrated that following asymmetric cell division of SOPs in *Drosophila*, Delta is concentrated in recycling endosomes in pIIb cells. In loss of function studies, expression of either *rab11* or *sec15* mutants, which function together to recycle proteins back to the cell surface, produce cell-fate transformations to a phenotype consistent with loss of Notch signalling, which is thought to be due DSL ligand inactivity[161, 162].

When Notch from the signal-receiving cell associates with the DSL ligand from the signal-sending cell, the endocytosis of the ligand on the signal-sending cell, known as transendocytosis, induces a physical force on the Notch receptor on the signal receiving cell[20]. This force destabilizes the non-covalent bonds of the Notch heterodimers structure exposing the ADAM cleavage site and allowing for proteolytic activation of the Notch

receptor[20]. The S2 cleavage site is located in the extracellular portion of the Notch C-terminal fragmen and is cleaved by the metalloproteases ADAM10/TACE in mammals, Kuzbanian in *Drosophila*, are responsible for t[163-165]. This S2 cleavage removes the ectodomain resulting in a membrane-anchored Notch form termed Notch extracellular truncation (NEXT).

It is then believed that NEXT must first be endocytosed before it can undergo the γ-secretase-mediated S3 cleavage in the transmembrane domain of the Notch receptor. The γ-secretase activity is provided by the protease presenilin[166]. It has been demonstrated that inhibition of NEXT endocytosis by using dominant-negative forms of either Dynamin2 or Eps15 blocks γ-secretase processing of NEXT[167]. It was also found that NEXT can be monoubiquitinated at a conserved lysine residue, and mutation of this lysine reduced both NEXT internalization and S3 cleavage [167]. NEXT endocytosis may also be required for activated presenilin to have access to the S3-cleagave site. It is thought that biologically active presenilin pools are located in intracellular compartments. It has been shown that large pool of active presenilin complexes are found in lipid rafts within the endosomal pathway[168].

The transcription factors of the conserved mammalian CBF1/*Drosophila* Su(H)/*C. elegans* LAG-1 (CSL) family are the primary nuclear effectors of Notch signalling. In the absence of Notch activation, the CSL proteins act as transcriptional repressors with other known co-repressors on Notch target genes[169]. In *Drosophila*, CSL promotes the recruitment of the Asf1 histone chaperone to silence many Notch genes[170]. Upon γ-secretase proteolytic cleavage by persenilin in the transmembrane domain of NEXT, the Notch intracellular domain (NICD) is released. The NICD fragment then translocate into the nucleus where it physically binds to CSL and, together with the co-activator Mastermind (Mam in *Drosophila* and mammals; LAG-3 in *C. elegans*), forming a transcriptionally active ternary complex[171, 172]. Once formed, the complex recruits general transcription factors, such as CBP/p300 and PCAF, and promotes chromatin acetylation and expression of the Notch target genes[173, 174].

Once generated, the NICD signalling fragment can no longer be regulated by ligand binding or other cell-surface events. Disassembly of the CSL/NICD/Mam ternary complex and signal attenuation is mediated by ubiquitination and proteosomal degradation. NICD is

first phosphorylated by cyclin-dependent kinase 8 in its C-terminal PEST domain and then targeted for degradation by the E3 ubiquitin ligase Fbw7 in mammals and SEL-10 in *C. elegans*[175, 176]. It is important to tightly controlled NICD turnover to prevent sustained signalling for an inappropriately long period or at an excessively high level. It has been shown that some cases of T-ALL have mutations that delete the Notch PEST domain, which lead to an inability to degrade NICD properly[177].

The Notch receptor can also be targeted for lysomal degradation through ubiquitination directly from the plasma membrane. The E3 ubiquitin ligases Suppressor of deltex (*Drosophila*), Itch (*mammals*), SEL-10 (*C. elegans*)[178], and Cbl (*Drosophila* and mammals)[179], have been identified that target non-activated Notch for degradation[36, 180]. Numb is a conserved membrane-associated protein that acts upstream of the γ-secretase cleavage to block Notch signalling[23]. In 3T3 cell culture assay, Numb was shown to interact with Itch to promote Itch-dependent ubiquitinylation, endocytosis and degradation of Notch1[181]. Numb interacts with both Notch and AP-2, thus helping to recruit the components of the clathrin-dependent endocytosis pathway to internalize the Notch receptor[23, 24]. In some Notch expressing tissues, the absence of Numb can induce an accumulation of the receptor at the cell surface and an overactivation of Notch. This can lead to an overproliferation of cells and a tumour-like growth of tissues[25, 26].

Ubiquitination of the Notch receptor can also act positively on Notch signalling and influence the endosome trafficking of the Notch receptor[182] (Figure 3.C.). Deltex (dx), an E3 ubiquitin ligase containing a RING domain, has been shown to physically associate with Notch and the β-arrestin, Kurtz in *Drosophila*, promoting internalization of the whole complex[183]. Loss-of-function of *dx* has no affect on mutant fly viability and fertility, however Notch-like patterning defects were observed, suggesting that in some tissues Notch signalling is activated in a Deltex-dependent manner[35]. Resent genetic studies on the Drosophila HOPS and AP-3 complexes revealed that they are required for Deltex-dependent, ligand-independent Notch signalling[184]. They help deliver intact, non-ligand-activated Notch to the limiting membrane of the lysosome. This leads to the accumulation of the Notch receptor, ectodomain shedding and/or degradation, and γ-secretase-mediated activation of the receptor[184]. The HOPS complex act in late endosome maturation and lysosomal fusion, while the AP-3 complex act in endosomal trafficking of proteins to the

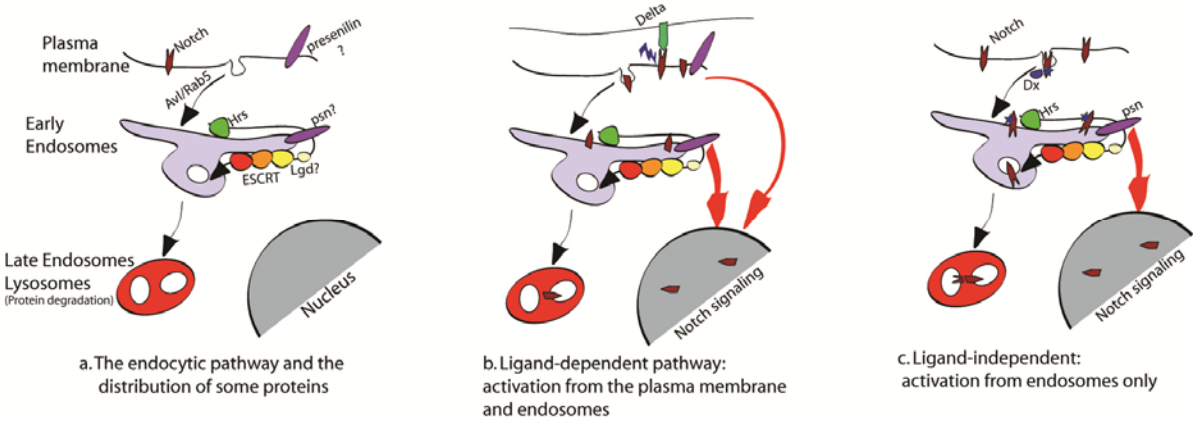


Figure 3: Models of Notch trafficking and activation in the endocytic pathway. a. Schematic representation of the main steps of the endocytic pathway. b. Schematic representation of the ligand dependent activation of Notch. Notch can be activated either at the plasma membrane or in the endosome. c. Schematic representation of the ligand-independent activation of Notch.

limiting membrane of the lysosome.

The ESCRT complex is another component that acts in ubiquitin-dependent endosomal sorting pathway that influence Notch signalling. *Drosophila* mutant cells for either *ept*, the drosophila homolog of tsg101, or *vps25*, two components of the ESCRT-I complex, undergo autonomous neoplastic transformation and also non-autonomous cell proliferation. This is due mostly to the activation of the Notch receptor that induces hyperproliferation of mutant cells as well as adjacent wild-type cells[27, 28, 185]. An increase of Notch signalling in mutant cells leads to the ectopic production of Unpaired, a ligand that activates the mitogenic JAK-STAT pathway in neighbouring wild-type epithelial cells. The Notch receptor accumulates in enlarged endosomes in *ept* and *vps25* mutant cells resulting in increased activation of Notch signalling[27, 28, 185]. RNAi knockdown of vps25 in *Drosophila* S2 cells increased S3 cleavage of Notch receptor by presenilin, an effect that was inhibited with the use of a presenilin inhibitor[185].

1.3.2. Notch and the hematopoietic system

During mouse embryogenesis, primitive hematopoiesis arise from mesodermal precursors, known as hemangioblasts, that migrate and become committed to endothelial and hematopoietic progenitors in the yolk sac blood island at embryonic day 7.5 (E7.5)[186]. Primitive hematopoiesis primarily consists of nucleated erythrocytes with embryonic-type globin. Hematopoiesis gradually shifts to the fatal liver after E11.5 and later to spleen and bone marrow[186]. Hematopoietic progenitors and lymphopoietic cells that will give rise to adult-type blood cells are detected in the paraaortic splanchnopleura region of mouse embryo at E7.5–9.5[187, 188]. The long-term repopulating hematopoietic stem cells (LTR-HSCs) that can reconstitute the adult mice hematopoietic system originates from the intraembryonic aorta, gonads, and mesonephros (AGM) region at E10.5–11.5[189-191]. In the adult mouse LTR-HSC can self-renew and give rise to short-term HSCs. These ST-HSCs self-renew for a short period of time and eventually give rises to non-selfrenewing multipotent progenitors (MPPs). Over time the MMPs lose their multipotent capacities and they become either common myeloid progenitors[192] or common lymphoid progenitors[193]. Common lymphoid progenitors (CLP) can

differentiate into B, T, natural killer and dendritic cells[193]. The common myeloid progenitors can mature to give rise to two other sets of progenitors: the granulocyte/macrophage progenitors and the megakaryocyte/erythrocyte progenitors[192]. Recently, circulating T cell progenitors has been identified in blood, suggesting that commitment toward the T cell lineage can occur extrathymically[194, 195].

The major progenitor source that enters the T cell pathway in adult mice is the lymphoid primed multipotent progenitors (LMPPs) found in both the bone marrow and peripheral blood. These cells can give rise to macrophages, dendritic cells, natural killer cells, B cells and T cells, but not erythrocytes or megakaryocytes[196, 197]. These cells migrate to the thymus, where the LMPPs are exposed to various factors that triggers and supports pro-T cell differentiation, proliferation and survival[198]. The earliest described intrathymic progenitors and are the early T-cells/thymic progenitors (ETPs), which are also known as $CD4^{-}CD8^{-}$ double-Negative 1 (DN1) cells. When sorted by flow cytometry (table 1) ETPs display a $Lin^{-}Sca1^{+}c\text{-}kit^{high+}CD24^{lo/+}CD25^{-}CD44^{+}$ surface phenotype[199, 200]. At this stage, DN1 retain a weak potential to differentiate into the B lineage potential both *in vivo* and *in vitro*, however they still have the ability to differentiate into natural killer cells and some dendritic and myeloid cells[201, 202]. DN2s ($c\text{-}kit^{+}DC44^{+}CD25^{+}$) retains some potential to differentiate into lineages other then T-cells[201]. True commitment to T lineage occurs only at the DN3 ($CD44^{-}CD25^{+}$) stage after completion of pre-T cell receptor (TCR) β rearrangement is completed (DN4) (figure 4) [203]. The TCR is specific to T cell and is expressed at the cell surface. It is generally responsible for recognizing antigens bound to major histocompatibility complex. DN4 then become mature $CD4^{+}CD8^{+}$ double-positive (DP) T cells. These DP T cells go on to differentiate into different subset of T cells, single positive (SP) $CD4^{+}$ or $CD8^{+}$ T cells[204].

The implication of Notch in the hematopoietic system was first discovered though the identification that Notch plays a critical function of in T lineage development. Neonatal mice that expressed a loss of function Notch1 had a severe deficiency in thymocyte development[205]. Also, in competitive repopulation of lethally irradiated wild-type mice bone marrow with wild-type- and Notch1-deficient bone marrow, a blockage in T cell development was observed at an early stage[205]. In a concurrent experiment, when the constitutively active form of Notch1 was expressed to reconstitute irradiated bone marrow,

the repopulated bone marrow was mostly composed of immature $CD4^+$ $CD8^+$ T cells and there appeared to be a simultaneous block in early B cell lymphopoiesis[206]. At the time, these results suggested that Notch1 was a key regulator in determining T lymphoid versus B lymphoid lineage[205, 206]. Subsequent studies have demonstrated that Notch signalling is important in many hematopoietic lineages and plays essential roles at several stages of T cell development, differentiation and function, including during T cell immune responses[207-209]. Notch signalling was also shown to be essential for the generation of foetal hematopoietic stem cells (HSCs), but appears dispensable for the maintenance of adult HSCs[210, 211].

Over the past decade, it has been demonstrated that Notch1 is a critical player in committing hematopoietic progenitors to the T cell fate. Common lymphoid precursors (CLP)s deficient for Notch1 develop into B cells[207, 213] and dendritic cells[214], while overexpression of Notch1 in common lymphoid precursors drive T cell development at expense of natural killer and B lymphoid cells[207], and the myeloid lineage[215]. Notch1 interacts specifically with Delta-like-4 (Dl4), which is expressed at high levels in thymic epithelial cells[216]. T cell development can be studied *in vitro* using a two-dimensional cultures system where hematopoietic progenitors are exposed to Delta-like ligands that are either coated on the plate or expressed by cells[212-214]. OP9 cells are a macrophage colony stimulating factor-deficient bone marrow stromal cell line that can support lymphoid lineage differentiation. Mouse foetal liver, bone marrow HSCs and embryonic stem cells, as well as human cord blood and bone marrow cells co-cultured on top of OP9 cells transduced with the Notch ligand Delta-like-1 (OP9-Dl1) cells supports T lymphopoiesis, while having a dose-dependent negative impact on B, natural killer, and monocytic/dendritic cell generation[215]. It has been demonstrated that in hematopoietic progenitors, Notch1signalling acts through the canonical CSL and Mam pathway to activate transcription[216, 217]. Progenitors cultured with ligands of the Jagged family are not driven to a particular pathway and thus develop into all hematopoietic lineages[213, 214].

Notch signalling intensity is carefully regulated throughout T cell development and differentiation (Figure 4). Notch signalling is maintained a very low level in primitive hematopoietic progenitors located in the bone marrow. In the bone marrow Notch signalling is inhibited by the transcriptional repressor Leukemia/lymphoma-Related Factor

Markers	Type of Cell	Lineage	Day of measurement (relative to the first day in culture)
$Lin^{-}Sca1^{+}c\text{-}cit^{high+}$ $CD24^{lo/+}CD25^{-}CD44^{+}$	ETPs (LSK-like)	B and T-cells	Day 0, 5 and 12
$c\text{-}kit^{+}$ $CD25^{+}$ DC44+	DN2	T-cells	Day 5
$c\text{-}kit^{-}$ $CD25^{+}$ $DC44^{-}$	DN3	T-cells	Day 5 and 12
$CD4^{+}$ $CD8^{+}$	DP	T-cells	Day 12
$CD4^{+}$ $CD8^{-}$ $TCR\beta^{+}$/ $CD4^{-}$ $CD8^{+}$ $TCR\beta^{+}$	SP	T-cells	Day 12
$B220^{+}$ $CD19^{-}$	pre-pro-B	B-cells	Day 5
$B220^{+}$ $CD19^{+}$	pro-B to mature B-cells	B-cells	Day 12
$CD11b^{+}$	Monocytes	Monocytes	Days 5 and 12
$NK1.1^{+}$	Natural killer cells	Natural killer cells	Days 5 and 12

Table 1: *In vitro* lymphopoiesis of HMCs by OP-9 co-culture and flow cytometry markers used to identify the different lineages

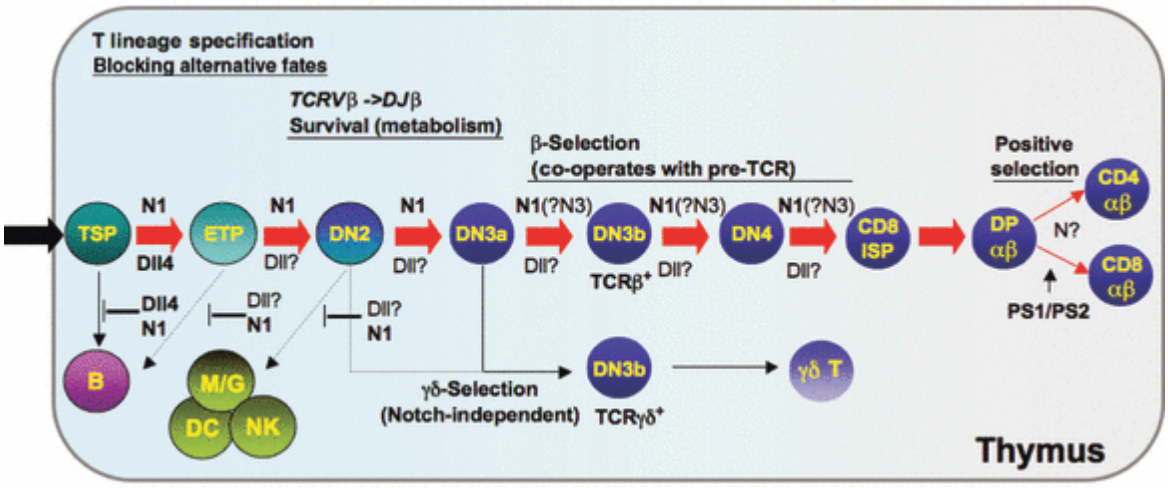


Figure 4: Notch signalling in T- and B-cell development. In the thymus, Notch1 (N1) in thymus-seeding progenitors (TSPs), which are most commonly lymphoid primed multipotent progenitors (LMPPs), interacts with Delta-like 4 (Dll4) on thymic epithelium to suppress their B-cell potential and promote T-lineage specification. N1 also suppresses myeloid (M/G), dendritic cell (DC), and NK-cell fates at the early T-cell precursor (ETP) and double-negative 2 (DN2) stages, but the Notch ligand required for this suppression *in vivo* has not been identified. N1 also regulates survival prior to β-selection, and then cooperates with pre-T-cell receptor (TCR) signalling to promote survival and proliferation during the DN3 to double positive (DP) transition. Figure adapted from *Stanley, P. and Guidos, C.J., 2009*[218].

(LRF)[219]. Knockout of LRF in hematopoietic progenitor bone marrow cells in mice was embryonic lethal due to severe anaemia. When these bone marrow LRF-negative cells were expanded in a co-culture system, the cells were no longer able differentiate into B cell, however they retain their ability to differentiate into T cell[219]. In a bone marrow repopulation assays, LRF-negative progenitor cells were unable to repopulate B cell linage cells in the peripheral blood, while the T cell population was relative normal compared to wild-type. Abnormally, lost of LRF in the bone marrow progenitors lead to extrathymic development of T cells in the bone marrow[219]. These experiments demonstrated that LRF acts in a cell-autonomous manner on hematopoietic progenitors, without altering Notch ligand expression in the bone marrow microenvironment[219].

The exact role Notch signalling in DN1-DN2 transition is ambiguous, however Notch signalling is required for the maintenance of CD25 expression in DN2 and DN3 cells and the survival of DN2, DN3 and DN4 cells[220, 221]. Notch signalling is maintained in mice until the β selection or pre-TCR checkpoint (Figure 4). Though Notch signalling is required for transition from DN4 to CD4+CD8+ double-positive (DP) cells, it is rapidly downregulated afterwards by pre-TCR signalling mechanisms[222, 223]. There are different subtypes of the TCR receptors. Mature T cells that select for the TCRαβ recognise peptidic antigens bound to major histocompatibility complex (MHC) proteins[224] and selection of which peptidic antigens the T cell will recognise occurs prior to leaving the thymus. Antigen selection for mature TCRγδ T cells occurs in the peripheral blood oppose as in the thymus. These TCRγδ T cells can be triggered to produce cytokines in their naïve state and do not require the MHC molecules for antigen selection[225]. In mice, selection for αβ over γδ T cell lineage is dependent on Notch signalling[203, 226]. The opposite is true in humans. High levels of Notch activation favour the γδ T cell lineage over the αβ T cell lineage[227].

The role of Notch signalling in the selection of mature signal positive (SP) $CD4^+$ or $CD8^+$ cells is controversial with gain- and loss-of-function experiments being at odds with one another. When the constitutive active form of Notch (NICD) was expressed in mice, decreased $CD4^+$ and increased $CD8^+$ SP thymocyte was observed[228]. However, when endogenous Notch signalling was inhibited in thymocytes, little or no effect on $CD4^+$ and $CD8^+$ SP development was seen[228]. Modulation of the downstream Notch effectors, such

as conditional deletion of CSL[229] or induction of a dominant negative form of Mastermind[230], in mice produced normal SP thymocytes. However when deleting Presenilin1/2, the mice show impaired SP thymocyte development[231]. Discrepancies between these results could be due to differential temporal expression of the various proteins. The gene deletion of Presenilin1 is initiated at the DN2-DN3 stage, thus occurs prior to TCRβ selection[231]. This severely impacts DP thymocyte number because the cells cannot mature past the DN3 stage[203, 232]. In these experiments, the deletion of the CSL gene is induced later, at the DP stage[231], while the mutant mastermind is not expressed until the DP stage. The fact that CSL and mastermind deficient thymocytes generate normal numbers of DP thymocytes [229, 230] indicates that Notch signalling is down regulated later, most likely after TCRβ selection.

Notch signalling and intensity must be careful regulated, since deregulation of Notch activation in T cells can give rise to T cell leukemia, both experimentally and in patients[233]. Notch was first implicated in T-ALL when a rare t(7:9) translocation between the TCRβ promoter/enhancer sequences and the genomic region encoding the NOTCH1 intracellular domain was discovered[16]. Since then, activating NOTCH1 point mutations have been described to occur in 50% of T-ALL cases[177].

1.3.3. Asymmetric cell division and Notch Signalling

Asymmetric cell division (ACD) is crucial for generating diversity. The underlying mechanisms of ACD has emerged over the past decade from studies performed in *Drosophila melanogaster and Caenorhabditis elegans* (figure 5). Asymmetric division occurs when a mother cell gives rise to two daughter cells with different fates. In some cases the two daughters are initially identical at birth and undergo the acquisition of different cell fate later on. Alternatively, the mother cell can become polarized and the two arising daughters receive different cell fate determinates at birth[148]. This is the case for dividing sensory organs precursor cells (SOP).

1.3.3.1. ***Asymmetric cell division of sensory organs precursor cells (SOP)***

The asymmetric division of SOPs yield two secondary precursor cells, the anterior cell, pIIb, and the posterior cell, pIIa. Cell fate specification comes from directional signalling between the two cells, where the Delta ligand secreted from the pIIb cell activates the Notch receptor on pIIa cells, thus initiating different transcriptional cell fate programmes in the two cells. For example, the mRNA of the transcriptional repressor tramtrack p69 (TTK69), is originally present in both cells[234, 235]. However, ttk69 translation is prevented in pIIb by the RNA-binding protein Musashi. In pIIa cells, Notch signalling prevents Musashi-dependent translational repression of *ttk69*, thus in the end, TTK69 is only present in pIIa cells[234, 235]. Another cell fate programs is the association of the activated NICD signalling fragment of the Notch receptor with Suppressor of Hairless to form a transcriptional activator complex in pIIa cells[236].

Originally Delta and Notch are expressed in both pIIb and pIIa. Directional signalling from pIIb towards pIIa is in part established through the asymmetric distribution Numb and Neuralized (Neur). During mitosis Numb co-localizes with Pins–Gαi at the anterior cortex of SOPs and thus is partitioned into pIIb[237, 238]. In *numb* mutants, two pIIa-like cells are generated giving rise to a Notch over expression phenotype, while overexpression of *numb* yields two pIIb-like cells that have Notch downregulation phenotype[239]. Numb associates with α-Adaptin, a component of the AP-2 complex that targets transmembrane proteins for endocytosis[23, 24]. Sanpodo, a transmembrane protein known to associates with Numb and to be implicated in Notch signalling[240], appears to also be involved in α-adaptin-mediated endocytosis[241]. In wild-type cells Sanpodo is present at the cell membrane of pIIa and localizes on endosomes in pIIb. In *numb* or *α-adaptin* mutants Sanpodo is no longer endocytosed and found at the plasma membrane of both pIIa and pIIb cells[241]. The E3 ubiquitin ligase Neur also regulates signalling between the pIIb and pIIa cells. Neur localizes at the anterior cortex of SOPs during mitosis and is inherited only by the pIIb[242]. *Neur* mutants inherit a Notch loss-of-function phenotype, thus indicating that Neur is needed for pIIa fate acquisition. Neur can monoubiquitylate Delta and promote its endocytosis into pIIb[242, 243].

The asymmetric distribution of Numb and Neur, while important, does not fully explain why signalling occurs only from pIIb towards pIIa. Differential distribution of the recycling endosome functions redundantly with Numb and Neur asymmetry[161]. The recycling endosome, which is marked by the GTPase Rab11, is found throughout pIIa. However, in pIIb cells, the recycling endosome is found clustered around the centrosome along with its binding partner, the Arfophilin homologue Nuclear fallout (Nuf)[161]. This clustering of the recycling endosome is believed promotes endocytosis and activation of Delta in the pIIb cells. Overexpression of *nuf* causes Rab11 clustering in pIIa. However simultaneous overexpression of *nuf* and the non-phosphorylatable form of Lethal (2) giant larvae (*lgl-3A)*, is needed to observed dramatic changes in cell-fate. Lgl is a cytoskeletal protein that recruits cell fate determinates to the cortex. When it is phosphorylated by aPKC, it dissociates from the actin cytoskeleton and excludes Numb and Neur from the posterior part of dividing SOPs[244]. Thus these results indicate that differential distribution of the recycling endosome functions in parallel with Numb and Neur asymmetry[161].

1.3.3.2. Asymmetric cell division of neuroblast

During Drosophila embryogenesis, neuroblasts originate from the neuroectoderm, a monolayer of epithelial cells, through a process of lateral inhibition[245, 246]. Specified neuroblasts delaminate from the epithelium layer and enter mitosis. During prophase two centrosomes migrate laterally to opposite sides of the neuroblast and align parallel to the epithelium layer[245, 246]. At metaphase the mitotic spindle is oriented along the apical-basal axis and upon cytokinesis, the neuroblast undergo asymmetric division that gives rise to a larger apical neuroblast and a smaller basal ganglion mother cell (GMC)[245, 246] (figure 5.b.). These two resulting daughter cells inherit different fates: the neuroblast can continue to divide asymmetrically and self-renew, whereas the GMC is committed to the differentiation pathway and divides terminally to produce two neurons or glial cells.

The apical-basal polarity of an epithelial cell is established through the evolutionarily conserved Par protein complex consisting of Bazooka (Baz, the fly homolog

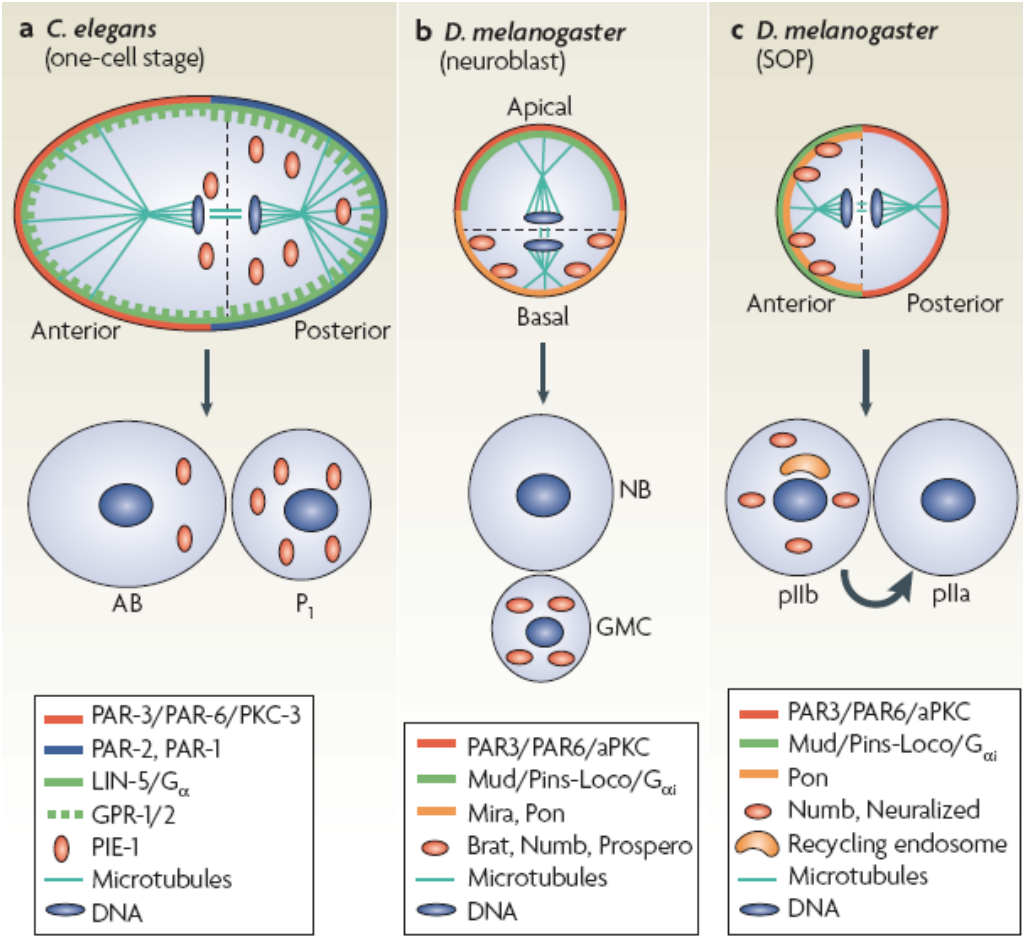


Figure 5: Asymmetric cell division in worms and flies. This figure depicting asymmetric division in one-cell *Caenorhabditis elegans* embryos (a), *Drosophila melanogaster* larval neuroblasts (b) and *D. melanogaster* sensory organ precursors (SOPs) (c) was taken from *Gönczy, P., 2009*[148]. The polarized mother cell during anaphase is shown at the top of each panel, while the resulting daughter cells are shown at the bottom. The distribution of important components for the establishment of polarity; spindle positioning and cell-fate determination is illustrated for the mother cell, and the distribution of cell-fate determinants is shown for daughter cells. Furthermore, directional signalling between the anterior cell (pIIb) and the posterior cell (pIIa), whereby the Delta ligand from pIIb signals to the Notch receptor on pIIa, is denoted by a thick arrow in panel c. GMC, ganglion mother cell; Mud, mushroom body defectice; NB, neuroblast.

of *C. elegans* Par-3), Par-6, and atypical PKC (aPKC) [245, 246]. When a neuroblast delaminates from the epithelium, it forms an apical stalk which remains in contact with the surrounding epithelial cells. The Par complex located in the apical stalk colocalizes with Inscuteable (Insc), a neuroblast-specific protein, and establishes the apical-basal polarity in the delaminating neuroblast[247-249]. During mitosis, the Insc/Par complex recruits a complex composed of Partner of Inscuteable (Pins) and the heterotrimeric G protein subunit $G_{\alpha i}$ to maintain apical-basal polarity at the apical cortex[250-252].

These two protein complexes have distinct roles. The Pins/$G_{\alpha i}$ complex is mainly involved in orienting the spindle perpendicular to the epithelium layer at metaphase[248, 253], while the Par complex primary plays role in the basal localization of cell-fate determinants. The apical Par complex regulates Discs large (Dlg) and Lethal (2) giant larvae (Lgl), two cortically localized tumour suppressors, to direct the basal localization and segregation of cell-fate determinants, such as Prospero (Pros) and Numb and their adaptor proteins, Miranda (Mira) and Partner of Numb (Pon)[244, 254, 255]. The PKinase aPKC inactivates Lgl by phosphorylation, thus preventing Lgl from associating with the apical cortex. However, phosphorylated Lgl can restrict myosin II activity to the apical cortex, resulting in expulsion of cell-fate determinants to the basal cortex[256]. In the basal cortex, Lgl is not phosophorylated and thus remains active and recruits cell-fate determinants to the basal cortex[244]. Myosin VI also positively regulates basal localization by recruiting Mira/Pros via vesicle transport machinery[257].

Notch signalling plays a role in regulating cell fate choice in the central nervous system. The selection of a single neuronal precursor from a cluster of equipotent progenitors that express the basic helix-loop-helix (bHLH) transcription factors, termed the proneural genes, is done through the Notch pathway. The expression of these proneural genes in the ectoderm is both necessary and sufficient to initiate the development of neuronal lineages and to promote the generation of progenitors that are committed to differentiation[258]. In loss-of-function experiment, when Notch signalling is lost, the cells within the clusters retain expression of the proneural genes and become neural cells[259, 260]. While the expression of the constitutively active form of Notch causes the cells within the cluster acquire an epidermal cell fate[261]. During the selection of neural precursors, in both *Drosophila* and vertebrates, the intracellular domain of Notch associates

with its co-activator Suppressor of Hairless (Su(H)) to elicits the transcriptional activation of the Enhancer of Split (E'(Spl)) family of bHLH transcriptional repressors. In turn, Enhancer of Split proteins repress the proneural genes[261-263]. This inhibition of proneural gene expression leads to the inhibition of a neuronal fate.

The neural precursor gene *asense* (*ase*), has long been accepted to be expressed in all neuroblasts [264, 265] and is often used as a neuroblast marker during immunohistochemistry studies. A recent study identified eight neuroblasts per brain lobe that do not express the Ase protein, which were named Posterior Asense-Negative (PAN) neuroblasts[266]. Six of the PAN neuroblasts are located medially and divide asymmetrically to produce Ase^- neuroblast and an Ase+ neuroblast. These Ase^- neuroblast are usually surrounded by long chains of Ase^+ progeny cells, typical Ase^+ neuroblasts usually have a small number of closely associated Ase^+ progeny[266]. The other two additional PAN neuroblasts generated progeny that populate interior brain regions[266]. The Ase^+ progeny cells continue to amplify transiently and eventually differentiate into neurons or gila cells.

It was found that expression of *brat* RNAi, a cell-fate determinant that is inherited in GMCs, causes defective differentiation in Ase^+ progeny of PAN-neuroblasts leading to cellular overgrowth[266]. Further investigation demonstrated that this overproliferation was due to over deregulation of the Notch pathway. *numb* loss-of function mutant, *lgd* mutant or overexpression NICD causes phenotypes similar to the *brat* RNAi phenotype[266]. Knockdown of *Notch* by RNAi had two different effects on PAN-lineages: it either eliminated them entirely or reduced the number of associated Ase^+ progeny[266]. These results suggest that Notch signalling is active and required for proper asymmetric division and differentiation in the PAN-neuroblast and the secondary Ase^+ neuroblasts. However, overactivation of Notch causes uncontrolled division of Ase^+ secondary neuroblasts[266].

1.4. Notch and mammalian brain development

The mammalian neocortex develops into a highly organized six layered structure at the anterior end of the neural tube. The first set of neural progenitor cells are found in the ventricular zone (VZ). After the onset of neurogenesis, a second germinal area, known as

the subventricular zone (SVZ), forms just below the VZ. Both the VZ and SVZ contain highly proliferative neural progenitors which can undergo symmetric and asymmetric cell divisions to either maintain the proliferative pool or produce the neurons of the different cortical layers[267]. Neurogenesis approximately begins at day 10.5 of embryogenesis (E10.5) in mice and lasts until around E17.5, when gliogenesis begins in the SVZ[268]. Newly produced neuronal progenitors in the VZ or SVZ migrate radially out of these zones along radial glia cells to the neocortical plate, where they differentiation and forms neuronal circuits[267, 269].

Evidence that Notch can inhibit neuronal differentiation in vertebrates was first seen in vitro study using embryonic carcinoma cell line P19 by *Nye et al., 1994*[270]. These cells can be induced to differentiate into neurons, astrocytes, or myoblasts under certain conditions[271-273] (figure 6.A). When *Nye et al., 1994*[270] stably transfected the murine full length Notch receptor or the active form, the NICD, into P19 cells, they inhibited the differentiation of the P19 cells into neurons and myoblasts[270]. However activation of Notch had no effects on astrocyte differentiation[270]. Since this initial study, the role of Notch signalling in the embryonic mammalian nervous system has been extensively investigated using both loss- and gain-of-function approaches.

Mouse embryos expressing Notch1 mutants were found to be embryonic lethal. Homozygous mutant embryos died around 11.5E[274, 275]. Morphological and histological analysis of the homozygous mutant embryos indicated that pattern formation through the first nine days of gestation appeared largely normal. However, mutant embryos at a later stage revealed widespread cell death[274, 275]. Detailed analysis of neuronal development of Notch1$^{-/-}$ mice was first carried by *de la Pompa et al., 1997*. They found that expression of either loss-of-function Notch or CSL caused reduced expression of the hes-5 target gene and an increase expression of Dl1[276]. They also found an up regulation of the early differentiating neurons markers Math4A, neuroD and NSCL-1 in the Notch1 and CSL mutant embryos, demonstrating that there was an excess of committed neuronal precursors generated at 9.0E in the mutants[276].

Mice carrying nulls for the other Notch alleles were also made. Embryos homozygous for either the Notch3 or Notch4 mutant developed normally and homozygous mutant adults were viable and fertile[277, 278]. There were no detectable genetic

interactions during early embryogenesis between the Notch3 mutation and a targeted mutation of the Notch1 gene when double homozygous mutant embryos were made[278]. However, the Notch4 mutation displayed genetic interactions with a targeted mutation of the related Notch1 gene. Embryos homozygous for mutations of both mutants displayed a more severe phenotype than Notch1 homozygous mutant embryos[277]. Both Notch1 mutant and Notch1/Notch4 double mutant embryos displayed severe defects in angiogenic vascular remodelling[277]. Notch2 homozygous mutant mice resemble Notch1 mutant mice the most. Notch2 deficient embryos die around 11.5E. Notch2 was found to be expressed in a wide variety of tissues including neuroepithelia, somites, optic vesicles, otic vesicles, and branchial arches, but not heart[279, 280]. Histological analysis of the mutant embryos showed earlier onset and higher incidence of apoptosis, particularly in neural tissues. However Notch2 mutant mice, in contrast to Notch1-deficient mice, do not show disorganized somitogenesis and the expression of the neurogenic genes such as Hes-5 or Mash1 remain unchanged[279].

Retroviral expression of Notch1 in neuronal precursors at E14.5 inhibits the generation of neurons, delays the emergence of cells from the subventricular zone (SVZ), produces an augmentation of glial progeny and postnatal astrocytes[281, 282]. When these $Notch^{+}$ neuronal progenitor cells were cultured for a short period of time, Notch1 inhibited their proliferation and caused a decrease in neuron generation (Figure 6.B)[281]. However in longer gliogenesis cultures, activated Notch1 triggered rapid cellular proliferation causing a significant increase in radial gial cell generation (Figure 6.B)[281]. Notch3 has been shown to be implicated in radial gial cell fate also. Retroviral infection at 9.5E of murin neuronal progenitors with the NICD3 promoted the generation of radial glial embryonically and astrocyte fate postnatally (Figure 6.B)[283]. Expression of an activated form of CBF1 (CBF1-VP16), a Notch effector, or of the Notch target gene, Hes5, also promotes radial glial cell fate in vivo[283]. This is very similar to what is seen in *Drosophila* neuronal development. Radial glia have their cell bodies in the VZ and extend radially to the pial surface, which cotes the surface of the cortex[284]. These cells are thought to provide a migratory scaffold along which newly generated neurons migrate from the VZ to postmitotic areas[285]. The migrating neurons express Notch ligands, such as

Delta. They promote radial glial identity by activating the Notch receptor expressed along radial glial fibbers[286].

1.5. The Drosophila l(2)giant discs (lgd) protein

The l(2)giant discs (lgd) protein was first discovered in a mutagenesis screen and was named after its ability to causes massive overproliferation of imaginal disc cells when non-functional[287]. This protein is evolutionary conserved and can be found from worms to mammals. However, it is not found in yeast[38]. Lgd encodes a 816 amino acid protein that contain four DM14 repeats of unknown function[288], three Lgd homology domains only found in this protein family, and a C-terminal C2 (Protein Kinase C conserved region 2) domain. C2 domains are known as calcium and lipid binding domains, and they are thought to have many functions such as protein-protein interaction, membrane recruitment, protein localization, and trafficking [289]. It was found that the lgd C2 domain binds preferentially to PI(3)P and PI(4)P, which are associated with the early endosomes and secretory vesicles[290], respectively, on a PIP strip suggesting that Lgd acts in the endocytic or secretory pathways[38].

Early characterization of *lgd* mutant wing discs found a deregulation of the wingless and decapentaplegic genes[291]. Wingless is one of the genes regulated by Notch at the dorso-ventral boundery during wing development. Notch over activation causes similar phenotypes as that of *lgd* mutants in the wing disc[292, 293] leading to speculation that the *lgd* mutant phenotype could stem from the ectopic activation of the Notch pathway. This was found to be indeed the case. Thomas Klein demonstrated that loss of *lgd* function leads to the ectopic expression of Notch target genes[40]. Ectopic expression of wingless and cell overproliferation was abolished in *lgd* and *presenilin* double mutant wing discs as well as in *Suppressor of Hairless* and *lgd* double mutants[39, 40]. These results indicated that presenilin acts upstream of lgd and that lgd helps in the processing of Notch. Later studies found that *lgd* mutant wing discs overgrowth was caused by cell autonomous ligand-independent Notch activation[37, 39]. This was due to the accumulation of Notch in subcellular puncta labelled by Hrs, an early endosome marker, resulting in ectopic Notch signalling[37]. Lgd appears to function prior to the ESCRT complex since *lgd* and *vps25*

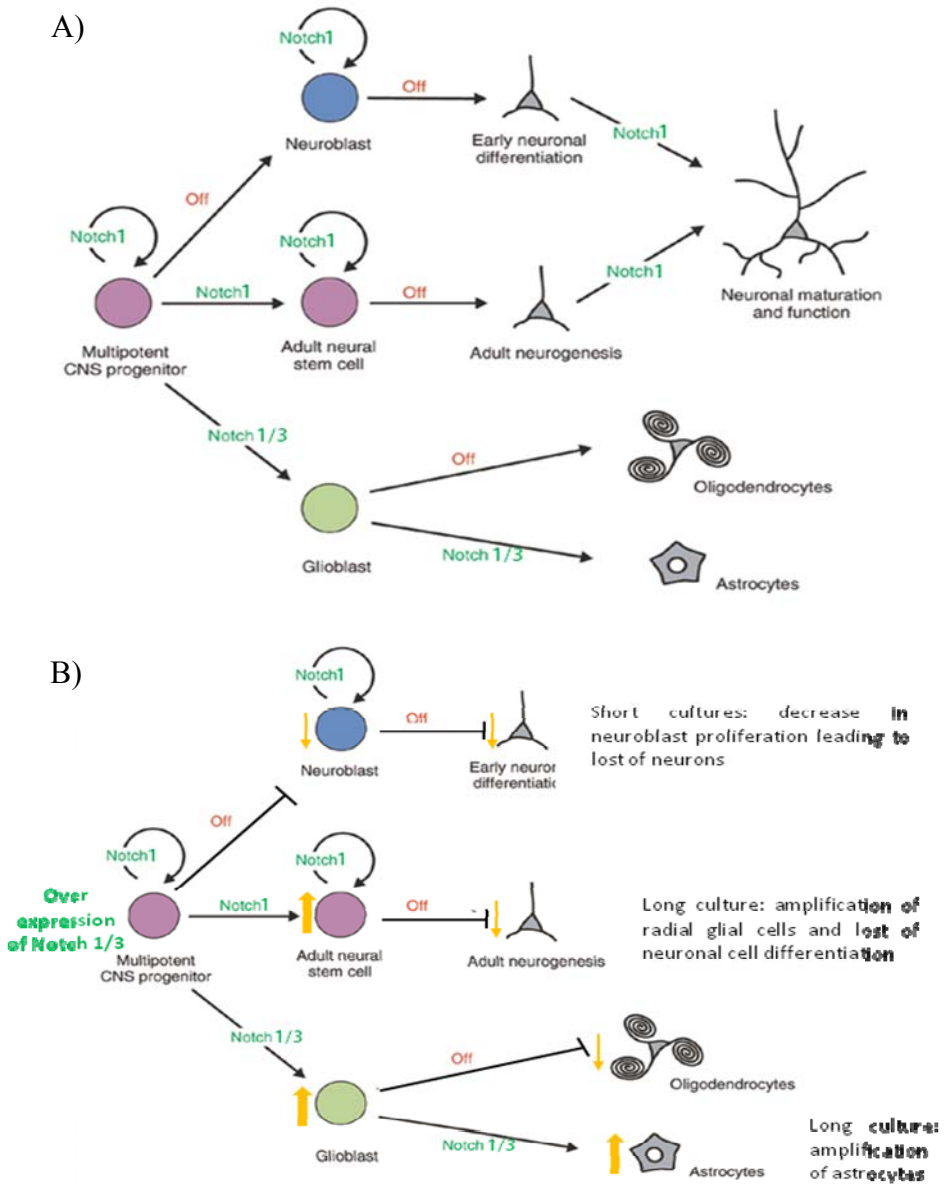


Figure 6: Notch signalling in the developing and adult central nervous system.

A) Processes that are likely to involve pathway signalling are labelled 'Notch' (green), and those that are likely to require downregulation of Notch signalling are labelled 'Off' (red).

B) Phenotype observed when isolated neuronal precursors that overexpress Notch1/3 are cultured. Figure was adapted from *Yoon, K. and Gaiano, N., 2005*[294].

double mutants do not alter or enhance the *lgd* mutant phenotype. However, ectopic Notch activation is no longer seen in *hrs* and *lgd* double mutants suggesting that lgd functions downstream of hrs[37].

Expression of mutant *lgd* or overexpression of full length *lgd*, also leads to Notch over activation in SOP. Notch and Delta in pIIa and pIIb cells are found in enlarged vesicles positive for Hrs and Rab5, makers of the early endosome, when lgd is overexpressed[38]. *Lgd* mutants had no affect on effect on the size, shape, or distribution of the late endosome, recycling endosome and the exocyst complex, indicating that lgd functions in the early endosome[38]. Lgd appears to be required for general protein sorting since disruption of *lgd* function leads to not only an accumulation of Notch in enlarges early endosome punctas, but also of ubiquitinated protein and of the *Drosophila* EGF receptor[38, 39]. Recent studies done in the *Drosophila* wing disc, has demonstrated that the two murine orthologs of lgd can rescue the *Drosophila lgd* mutant and restricts the expression of wingless to its normal domain along the dorso-ventral boundary.[39]

1.6. The mammalian lgd orthologs, coiled-coil and C2 domain containing 1 (CC2D1) protein A and B

Very little is known about the mammalian counterparts of *lgd*. The human CC2D1A, which will be referred as hlgd1, was first found to be involved in autosomal recessive inheritance of non-syndromic mental retardation (NSMR)[295, 296]. Mentally retarded individuals affected do not have major physical abnormalities, dysmorphism, or neurological abnormalities. It is thought that biological processes involved in neuronal differentiation, synaptic plasticity, synaptic vesicle cycling, and gene expression regulation are the causes of mental retardation[297]. Homozygosity mapping of individuals affected by NSMR of the same family identified a homozygous and haploidentical region of 2.4 Mb to 0.9 Mb on chromosome 19p13.12[295, 296]. Sequencing of the cDNA with various primers found different types of mutations. The first mutation mapped was the complete deletion of 3589 nucleotides beginning in intron 13 and ending in intron 16 of *hlgd1* [295]. The deletion caused a frame shift immediately after the deletion, creating a nonsense

peptide of 30 aa and a stop codon at position 438 of the mutant protein[295]. The mutation caused a loss of the DM14 and C2 domains of the gene. Western blot analysis done in lymphoblastoid cells derived from patients using a CC2D1A antibody, which detected the N-terminus of the protein, identified a 104 kDa band in normal individuals but not in the patients[295]. Mapping done on another family found a splice mutation that result in the skipping of exon 19[296]. The splicing of exon 18 to exon 20 causes a frameshift, resulting in the addition of 13 nonsense amino acids after exon 18 and the addition of a premature stop codon. The protein is truncated after the amino acid 740 resulting in the lost of the C2 domain[296].

In situ hybridisation studies were done to examine spatial expression patterns of mlgd1 during murin brain development. It was observed that at E12 the mlgd1 mRNA was expressed throughout the ventricular zone, developing cortical plate and ganglionic eminences[295]. By E16, mlgd1 mRNA was expressed throughout the mouse brain, and was particularly expressed strongly in the cortical plate[295]. At postnatal day 3, mlgd1 mRNA continued to be widely expressed with strong expression in the cerebral cortex and hippocampus[295]. The mlgd1 mRNA was expressed in the brain into adulthood[295]. Given the expression patterns of mlgd1 and that mutations in the hlgd1 gene leads to poorly differentiated neurons in NSMR, it appears that mammalian lgd is implicated in brain development.

Subcellular localisation studies performed in the human osteosarcoma cell line U2OS using a polyclonal antibody for the hlgd1 protein, found that the protein was distributed throughout the cytoplasm and no signal was detected in the nuclei[295]. However, a study done by *Ou, X.M. et al, 2003* suggest otherwise. In a yeast one-hybrid assay to find regulatory proteins that target the serotonin receptor (5-HT1A) dual repressor element (DRE) using a murin brain cDNA library, *Ou, X.M. et al, 2003* identified mlgd1 as a transcription regulator for 5-HT1A[298]. In a luciferase assay using one of the DRE components, the 5'-repressor element, they demonstrated that over expression of m*lgd1* caused a decrease expression of the luciferase gene, while co-transfection with anti-sense cDNA increased the expression of the luciferase gene[298]. Western blot analysis of 5-HT1A receptor protein levels mirror the results obtained in the luciferase assay. Protein levels of 5-HT1A decreased after transient transfection with sense mlgd1 and increased

when anti-sense mlgd1 was transfected[298]. Furthermore, immunofluorescent staining of cells in culture and rat brain sections, *Ou, X.M. et al, 2003* showed that rat lgd1 (rlgd1) protein was present in the nucleus of cells. Cells that stained weakly for rlgd displayed stronger 5-HT1A staining, and vice-versa for cells expressing higher levels of rlgd1 [298]. This suggested that lgd1 protein inhibits the expression of the 5-HT1A receptor *in vivo* as well. The same group also showed that both human lgd othologs can also bind to the 5-HT1A DRE and represses the human 5-HT1A receptor gene[41].

A recent study by *Nakamra, A. et al, 2008* has implicated hlgd1 in the phosphoinositide 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt pathway. Using a HeLa cDNA library and a human foetal brain cDNA library they found that hlgd1 interacts with PDK1 in an *E. Coli* two-hybrid screen[42]. This interaction was confirmed in mammalian cells and co-immunoprecipitation studies with various hlgd1 deletion mutants found that that the region around the forth DM14 domain is needed for hlgd1 to bind PDK1[42]. Akt activity is correlated with its phosphorylation levels at the Thr^{308} and Ser^{473} residues. It was found that down regulation of hlgd1 expression with siRNA causes a significant decrease in the levels of phospho-Thr^{308}-Akt, but not Ser^{473}[42]. GST pull-down analysis with *in vitro*-translated recombinant hlgd1 and Akt demonstrated that hlgd1 forms a complex with Akt and PDK1 and positively regulate PDK1-mediated Akt phosphorylation[42].

Several growth factors trigger the activation of PI3K, which leads to Akt activation. EGF stimulation increased the phospho-Thr^{308}-Akt levels. Knowdown of hlgd1 inhibits phosphorylation and activation of PDK1 induced by EGF stimulation[42]. It was found that hlgd1 could co-immunoprecipitate with the EGFR when cells were stimulated with EGF[42]. It was also demonstrated endogenous Akt and hlgd1 could form a complex upon EGF treatment, thus indicating that hlgd acts as a scaffold to form a multiprotein complex in response to EGF stimulation. In cooperation with PIP_3, hlgd1 recruits the PDK1/Akt signalling module to the activated EGFR[42]. It was also demonstrated that down regulation of hlgd1 by siRNA causes increased cellular apoptosis and sensitivity to chemotherapeutic drugs[42]. These results indicate that hlgd1 is regulator of PDK1/Akt signalling in the maintenance of cell survival and cell proliferation.

Materials and Methods

2.1. Fly Strains

Fly strains used were: InscGal4; UAS CD8::GFP/cyo (gift from JA Knoblich, Institute of Molecular Biotechnology of the Austrian Academy of Sciences, Vienna, Austria), UAS $Rab5^{QL}$/TM (gift from Marcos González-Gaitán, University of Geneva) , Ubx flp; lgd08 FR40 tubE GFP/cyo (gift from JA Knoblich). Crosses were preformed and tansgenes were expressed at 25°C.

2.2. Tissue staining and antibodies

Third-instar larvae were selected and brains dissected in PBS (Bioshop, Burlington, Ontario, Canada), fixed in 5% paraformaldehyde (Bioshop) in PBS for 20 min, and blocked using 2% BSA (Invitrogen, Carlsbad, California, USA) in PBS with 0.05% Triton X-100 (Bioshop) and 0.04% sodium azide (Bioshop). Brains were incubated with the primary antibody rat anti-Elav (1:300, Developmental Studies Hybridoma Bank (DSHB), Iowa City, Iowa, USA) guinea pig anti-Dpn (1:1000, gift from JB Skeath, Washington University School of Medicine, St Louis, Missouri, USA) and mouse anti-Notch intracellular domain (C17.9C6) (1:100, DSHB) overnight. Secondary antibodies used were anti-guinea IgG pig Alexa Fluor 555 (1:500, Invitrogen) and α-mouse CY5 (1:500, Invitrogen). Dapi (1:10000, Sigma-Aldrich, Oakville, Ontario, Canada) was used to stain nuclei. Brains were mounted in Mowiol 4-88 (Sigma-Aldrich).

2.3. Cell culture

The HEK-239T cells (Sigma-Aldrich), Hela (gift from PS Maddox, Université de Montréal, Montréal, Québec, Canada) and NIH-3T3 (gift from G. Sauvageau, Université de Montréal, Montréal, Québec, Canada) were maintained in Modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 100 U/mL penicillin and 100μg/ mL streptomycin (all from Invitrogen). OP9-GFP and OP9-Dl1 (gifts from C. Perreault, Université de Montréal, Montréal, Québec, Canada) were maintained in α-Minimal Eagle's medium (α-MEM) (Invitrogen) supplemented with 20% (FBS), 100 U/mL penicillin, 100μg/ mL streptomycin and 2 mM L-glutamine (Sigma-Aldrich).

2.4. Endocytosis Assay

Hela cells were plated onto 12 mm cover glass (VWR, Montreal, Quebec, Canada) at a density of $5x10^5$ cells/mL. Cells were allowed to adhere to the cover glass overnight. To label the early and late endosomes, the cells were incubated with 3 mg/mL rhodamine-dextran (10 KDa, Molecular probes-Invetrogen) for 15 or 45 minutes, respectively, at 37°C. Cells where then placed at 4°C, washed with PBS and fixed in 3% paraformaldehyde. Cells were permabilized with 0.1% Saponin (Bioshop) and then incubated with the primary antibodies mouse anti-EEA1 (1:50, BD laboratories, Mississauga, Ontario, Canada), mouse anti-LAMP2 (ABL-93) (1:20, DSHB), mouse anti-hLgd1 (1:250, Abcam Inc., Cambridge, Massachusetts, USA) and rabbit anti-hLgd2 (1:250, Aviva Systems Biology, San Diego, California, USA). Secondary antibodies used were anti-rabbit IgG Alexa Fluor 488 (1:500, Invitrogen) and α-mouse CY5. Dapi (1:10000) was used to stain nuclei. The cover glasses were mounted using Mowiol 4-88 onto cover slides (WVR).

2.5. Image acquisition

Images from fixed tissues and cells were acquired using a LSM 510 META inverted confocal microscope (Zeiss, Toronto, Ontario, Canada) using either a 40x or a 63X objective. Images were acquired by sequential scans in multiple channels. For figure assembly, images were processed with Photoshop (Adobe, Ottawa, Ontario, Canada), using only the "Gaussian blur" and the "level" function. Quantifications of vesicles were performed on the original images using the Image J program (National Institutes of Health (NIH), Bethesda, Maryland, USA).

2.6. Lentiviral production and infection

Five short hairpin RNA (shRNA) sequences specific to the mLgd1 and mLgd2 were designed (sigma-Aldrich) and cloned into lentiviruses.

mLgd1: 1) AGGCCCTAGAGAAACTGAAAG	mLgd2: 1) TGAGGATCGGGAAGAGATTTG
2) AGGGCAACATCATCAATGAAG	2) ATCGCATTGCCAAGCAATATC

3) ACTCCTAAGAAGCAGAATACC	3) GAGGCTCGGAAACTGCAATAC
4) TCCGCTTTGACTTCCCTTATC	4) ACCCTCCTAGTCATCACTTTG
5) GAGCGGCTAGAAAGGAAGATC	5) TGGCACAGCACACTTGAAATT

Briefly, the viral packaging and RNA polymerase III promoter plasmids (pMD2-VSVG, pMDLg/pREE and pRSV-REV, gift from S. Meloche, Université de Montréal, Montréal, Québec, Canada) were co-transfected by calcium phosphatase (chemical for solution from Bioshop) with the shRNA sequences into HEK-239T. Viral soup was collected after 48 hrs and filtered (0.45 µm, VWR). A 7 day puromycine (1 µg/ml, Invitrogen) kill curve using Hela cells was done to determine the viral titter. 12 hours prior to infection, 50 000 NIH-3T3 cells were plated in a 6-well plate. The medium was removed and replaced by the viral soup, calculated so that the multiplicity of infection (MOI) is 25. 7 µg/mL of polybrene (Invitrogen) was also added to the viral soup and cells were incubated for 36 hours. Cells were then transferred to a 10 cm dish and cells were challenged with puromycine (1 µg/ml) for 4 days for selection of cells expressing the lentiviruses.

2.7. RNA extraction, reverse transcription and RT-qPCR

Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Purified RNA was reversed transcribed using SuperScript™ II Reverse Transcriptase (Invitrogen) with random primers (Applied Biosystems) as described by the manufacturer. Expression level of target genes was determined using primer and probe sets from Universal ProbeLibrary: https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp.

Primer sequences are: mLgd1forward- gcctctcgcaatggactg, reverse-gcaccagcacaaagtcgtc; mLgd2 forward-ccagggtgctgagactgc, reverse-agcatgtcctcagggttgaa. Pre-developed TaqMan® assays for Hptr were used as endogenous controls. RT-qPCR analyses were performed as described using a PRISM® 7900 HT Sequence Detection System (Applied Biosystems, Streetsville, Ontario, Canada). The relative quantification of target genes was determined by using the ΔΔCT (cycle threshold) method. Relative

expression (RQ) was calculated using the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems) and the formula RQ = 2-ΔΔCT.

2.8. Western blot

Cells were harvested and lysed in NP-40 lysis buffer (Sigma-Aldrich). The lysates were cleared by centrifugation and the protein content was measured by the Bradford method (Biorad, Mississauga, Ontario, Canada). 50 µg of protein samples were resolved by 10% SDS-PAGE (reagents from Bioshop) and immunoblotted with the following antibodies: rabbit anti-β-actin (1:5000, Sigma-Aldrich), mouse anti-hLgd1 (1:1000), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:2500, Cell Signaling Technology, Beverly, Massachusetts, USA), and HRP-conjugated goat anti-mouse IgG (1:2500, BD Pharmigen, San Diego, California, USA). Chemiluminescent signal was detected using a LAS3000 imaging system (Fujifilm, Tokyo, Japan).

2.9. T-cell differentiation assay

E14.5 FL cells harvested from pregnant C57BL/6 mice (obtained from C. Perreault's lab) were prestimulated for 24 hr in DMEM containing 15% foetal-bovine serum, 6 ng/mL murine interleukin (IL)-3, 100 ng/mL murine Steel factor, and 10 ng/mL human IL-6 (All IL from PeproTech Inc., Rocky Hill, New Jersey, USA). FL cells were then separated into 6 groups. 5 of the groups were infected with different lentiviruses with 6 µg/mL of polybrene for 48 hr. Using a co-culture medium consisting of αMEM, 10% FBS, 10 mM HEPES (Invetrogen), 1 mM Sodium Pyruvate (Invetrogen), 50 µg/mL L-gentamycin, 55 uM 2- beta mercaptanol (Sigma-Aldrich), 100 U/mL penicillin, 100µg/ mL streptomycin, 1 ng/mL IL-7 (PeproTech Inc.) and 5 ng/mL Flt-3 (PeproTech Inc.), cells were then plated onto OP9-GFP expressing cells to stimulate B-cell differentiation or onto OP9-Dl1 to stimulate T-cells differentiation. Cells were collected on day 8, 12 and 16 and FACS sorted to determine their stage of differentiation.

2.10. Flow-Cytometry Analysis and Cell Sorting

The following antibodies were used: APC anti-mouse CD19, PE-Cy 5 Rat anti-mouse CD8a, APC Hamster Anti-mouse TCRβ chain, PE-Cy 7 Rat anti-mouse CD117 (c-Kit), APC-Cy 7 Rat anti-mouse CD4, PE anti-mouse CD25 (IL-2R) (Cedarlane Laboratories, Hornby, Ontario, Canada). All anti-bodies were from BD PharMingen unless indicated. Cells were analyzed on an LSRII flow cytometer with DiVa software and sorted on a three laser FACSAria (BD Biosciences).

Results

3.1. Perturbations of dlgd induce stem-cell tumours in the larval brain which resembles Notch over expression phenotype

Early characterization of *lgd* mutant wing discs found that loss of *dlgd* function leads to the ectopic activation of the Notch pathway [40]. Subsequent studies in both the wing disc and SOPs indicated that *dlgd* functions in the early endosome and appears to be required for general protein sorting[37-39]. In a recent study, it was found that early endocytic protein rab5 is also involved in Notch activation. Overexpression of a dominant active form of rab5 ($rab5^{QL}$) causes accumulation of the Notch receptor in the early endosome, overactivation of the Notch signalling pathway and overproliferation of cells in the wing disc[299]. To determine if perturbations of the endocytic pathway also affects Notch signalling during *Drosophila melanogaster* brain development, the $rab5^{QL}$ and the $dlgd^{08}$ loss-of-function mutant were expressed in neuroblasts using the *insc-Gal4* driver. Third-instar larvae were selected, brains isolated and immunostained with anti-Deadpan (Dpn, red) to label neuroblasts and anti-Elav (green) to label neurons (Figure 7). Expression of both $rab5^{QL}$ and $dlgd^{08}$ induces a dramatic overproliferation of neuroblasts compared to wild-type (WT). These overproliferations appear to originate from the posterior area of the brain that contains the 8 asense-negative PAN neuroblasts. When notch is overactivated in these cells, it causes uncontrolled division of Ase^{+} secondary neuroblasts[266] (Figure 7.B). This suggests that $dlgd^{08}$ induces Notch overactivation in PAN neuroblasts.

3.2. Expression of $dlgd^{08}$ causes vesicular accumulation of Notch in neuroblasts

Since overexpression of $rab5^{QL}$ causes the accumulation of the Notch receptor in the early endosome in the wing disc[299], it was next determined if *lgd* loss-of-function ($dlgd^{08}$) also results in Notch accumulation in the endocytic compartment. Staining of control and $dlgd^{08}$ mutant third-instar larvae brains for Notch revealed a clear accumulation of the Notch receptor in cytoplasmic vesicles (Figure 8.A.). The number of Notch positive

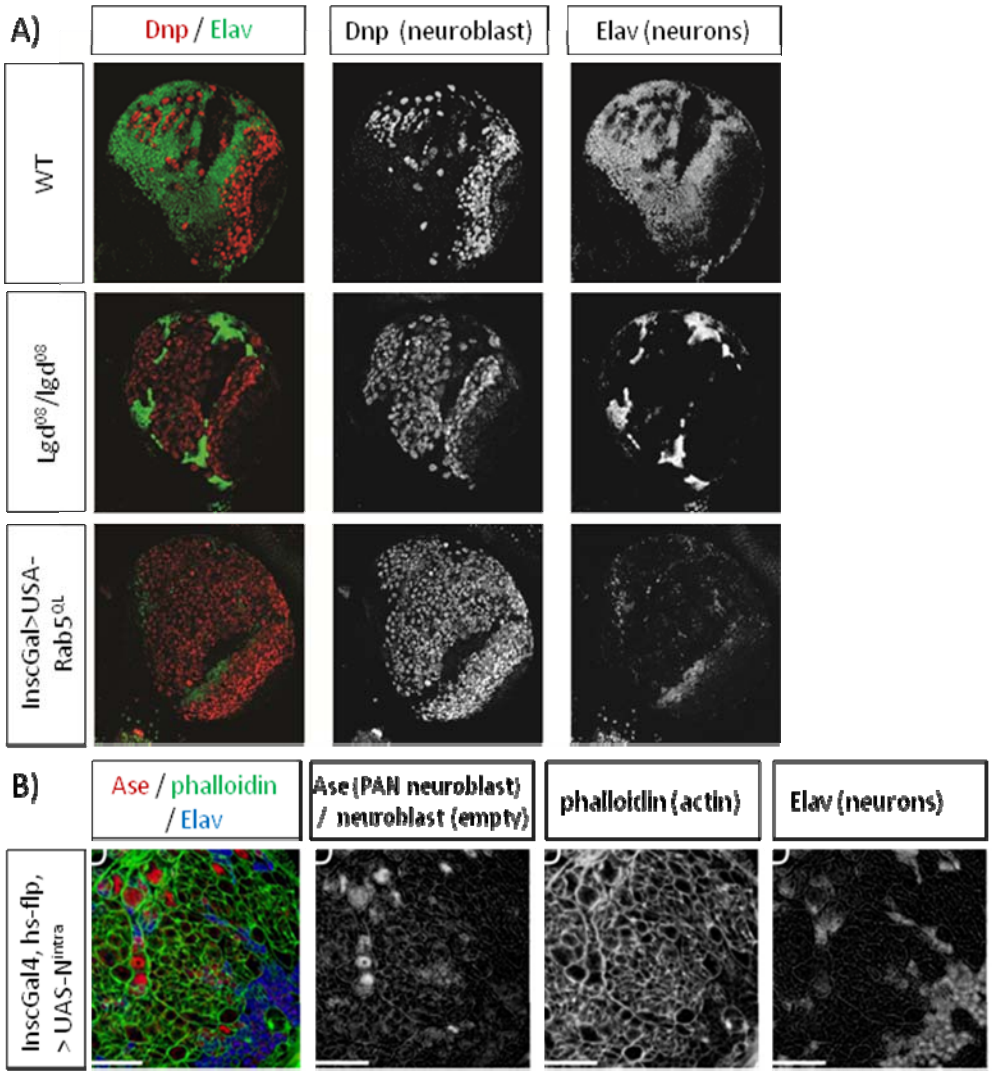


Figure 7: Perturbations of the endocytic pathway induce stem-cell tumors in the larval brain which resembles Notch over expression phenotype. A) Immunostaining of control (top panels), *lgd*^{08} mutant (middle panels) and Rab5^{QL} expressing (lower panels) third instar larvae brains. Brains were stained with anti-Dpn (red, marking neuroblasts) and anti-Elav (green, marking neurons). Note the reduction of the number of differentiated cells (neurons) in *lgd*^{08} and Rab5^{QL} brains. B) Immunostaining of N^{intra} expressing third instar larvae brains. Figure taken from *Bowman et al., Dev Cell 2008[266]*. Brains were stained with anti-Ase (red, marking PAN-neuroblasts), anti- phalloidin (green, marking actin) and anti-Elav (blue, marking neurons). Non-stained cells (cells delineated by actin, but are black) are neuroblasts.

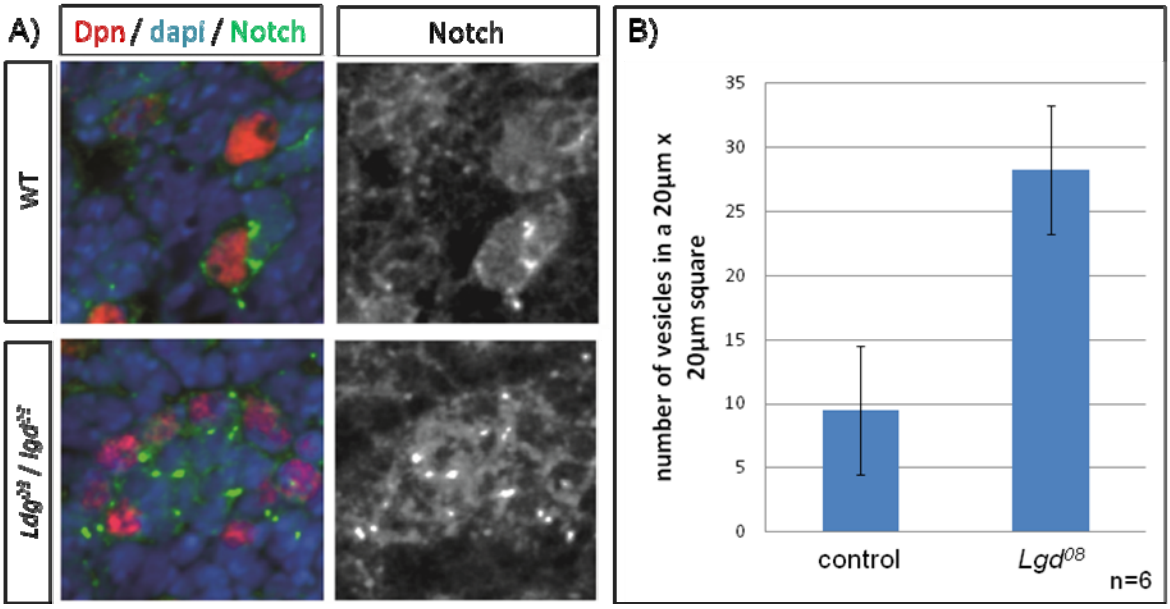


Figure 8: Vesicular accumulation of Notch. Notch localization in control (A) top panels) or *lgd* mutant (B) bottom panels) third instar larvae brains. (Staining: DAPI marks nuclei, blue; anti-Notch intracellular domain, green and anti-Dpn, red). B) Quantification of the number of Notch positive vesicles in the control and *lgd* mutant brains. In a 20µm x 20µm square, there were 9.5±5 vesicles in the control, and a three-fold increase of vesicles, 28.3±5 vesicles, in the lgd^{d08} brain. (n=6)

vesicles in the control and *dlgd*^{08} mutant brains were quantified (Figure 8.B.). A three-fold increase in the number of Notch positive vesicles in *dlgd*^{08} mutant neuroblasts was found compared to WT neuroblasts (number of vesicles in a 20μm x 20μm square: control=9.5±5, *dlgd*^{08} =28.3±5, n=6). These results are similar to what was observed in SOPs[38]. The fact that Notch accumulates in vesicles and it appears to be overactivated in these endocytic mutants, strongly suggest that Notch is activated from within endosomes in the *Drosophila* neuroblasts.

3.3. Proximal co-localization of hLgd1 with the early endosome and hLgd2 with the late endsome

Very little is known about the mammalian lgd1 and 2. It has been shown that both the murine *mlgd1* and *2* are able to rescue the *dlgd* loss-of-function Notch oveactivation and wing disc overproliferation phenotypes in *Drosophila*, thus demonstrating a conservation of function between homologs[39]. However, recent publications suggest that they may act differently in mammals than in *Drosophila*[41-44]. In mammals, published data shows or strongly suggest that the lgd homologs are not endocytic proteins but nuclear[41, 43, 44].Thus it was of interest to determine whethert the human orthologs hlgd1 and 2 were located in the endocytic compartment or in the nucleus. An endocytosis assay was performed. Hela cells were incubated with rhodamine-dextran (red) for 15 or 60 minutes to label the early and late endosomes, respectively. Cells were then fixed and stained with anti-hLgd1 (blue) and anti-hLdg2 (green) (Figure 9.A.). Both hLgd1 and hLdg2 can be found in distinctive punctea located in the cytoplasm, and not in the nucleus as previously observed[41, 298]. Though there is some co-localization between all three, the endosome, hLgd1 and 2, it appears that hLgd1 and 2 are localized in different compartments (Figure 9.A.). hLgd2 does not seem to co-localize with the early endosome. There was very little co-localization between the early endosome (red), the early endocytic maker early endosome antigen 1 (EEA1, blue) and hLdg2 (green) (Figure 9.B.). However it seemed to co-localize with the late endosome. There was a greater co-localization between the late endosome (red), the lysosomal marker lysosomal-associated membrane protein 2 [300] (LAMP2, blue) and hLdg2 (green) (Figure 9.C.). There were also some structures

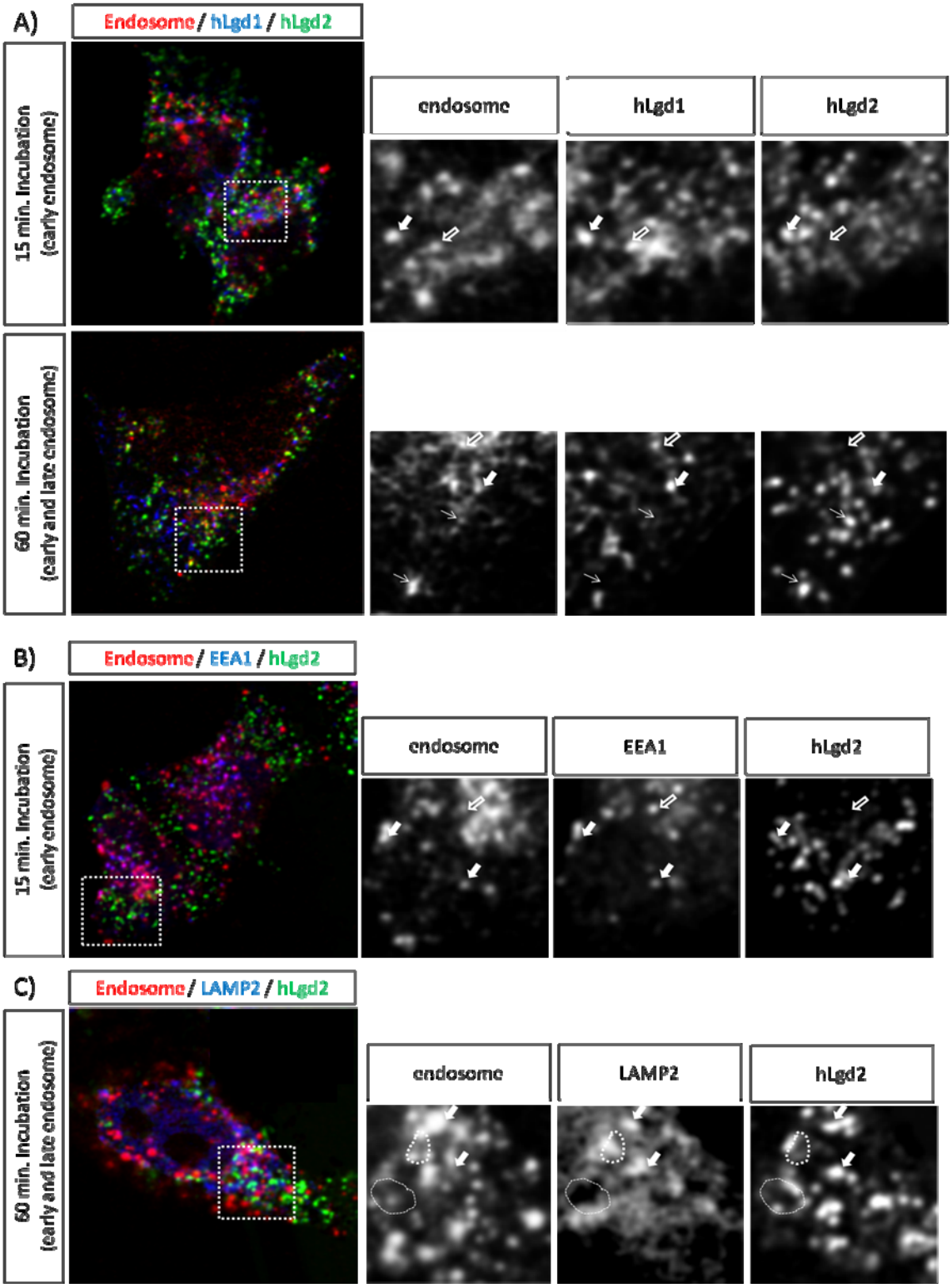

A)
Endosome / hLgd1 / hLgd2
15 min. Incubation (early endosome)
60 min. Incubation (early and late endosome)
endosome
hLgd1
hLgd2
B)
Endosome / EEA1 / hLgd2
15 min. Incubation (early endosome)
endosome
EEA1
hLgd2
C)
Endosome / LAMP2 / hLgd2
60 min. Incubation (early and late endosome)
endosome
LAMP2
hLgd2

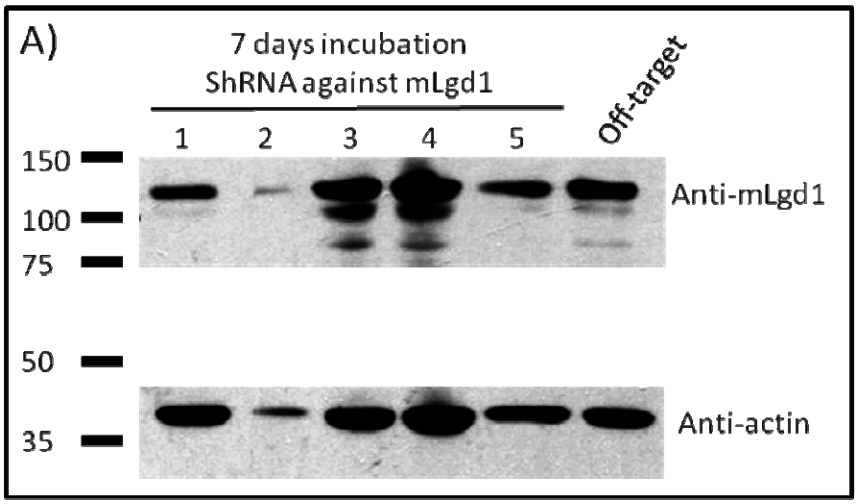
Figure 9: Proximal co-localization of hLgd1 with the early endosome and hLgd2 with the late endsome. Hela cells were incubated with rhodamine-labelled dextran for either 15 minutes, to label the early endosome, or 60 minutes, to label both the early and late endosome. A) Immunostaining with anti-hLgd1 (blue) and anti-hLgd2 (green) revealed that though there is some co-localization of both proteins (➧) with the early and late endosomes (red), the hLgd1 (⇨) and hLgd2 (→) proteins appear to co-localize with different endocytic compartments. B) Immunostaining with anti-hlgd2 (green) and the early endocytic marker EEA1 (blue). There was very little co-localization (➧) between hLgd1, EEA1 and the early endosome (red). C) Immunostaining with anti-hLgd2 (green) and the late endocytic marker LAMP2 (blue). There was co-localization between LAMP2 and hLgd2 and the late endosome (➧).There was also some structures where both hLgd2 and LAMP2 encircled the endosome (- - -) and structures where the endosome and hLgd2 ringed LAMP2 vesicles (**-...**).

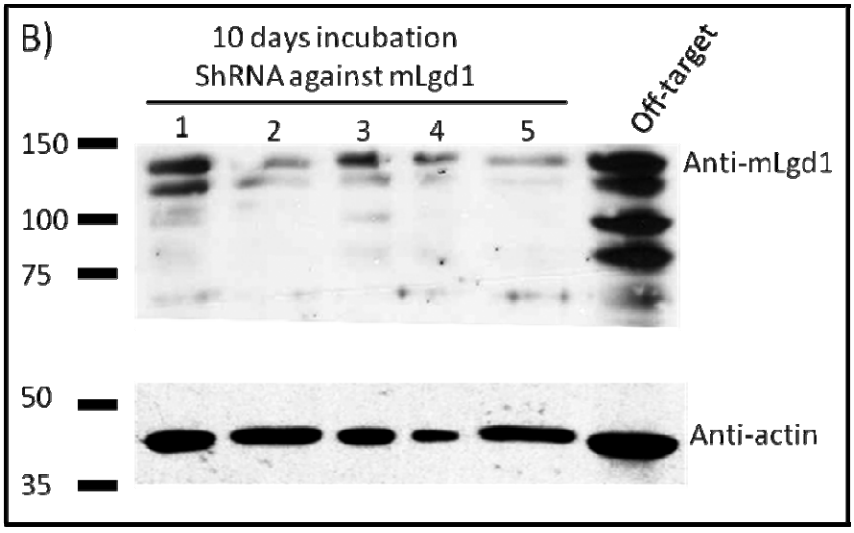
where both hLgd2 and LAMP2 encircled the endosome and structures where the endosome and hLgd2 ringed LAMP2 vesicles (Figure 9.C.), which are representative of lysomic vesicles[301]. These results suggest that hLgd1 is associated with the early endosome while hLgd2 is associated with the later endosome and lysosome.

3.4. Lentivirus production and knockdown of the murine mlgd1 and mlgd2 mRNA and protein levels

The next aim of the study was to determine if the mammalian Lgd also regulates Notch signaling in mammals. One of the best studied Notch dependent events is the differentiation of the T cell lineage. Notch signalling has been demonstrated to play essential roles at several stages of T cell development, differentiation and function, including during T cell immune responses[207-209]. Thus to best study the effects of mammalian lgd, it was decided to knockdown the murine mlgd1 and 2 in hematopoietic stem cells (HSCs) and observe how it affects T cell differentiation using a *ex vivo* OP9 co-culture T cell differentiation assay[215]. Primary cell lines and stem cells, which are long-term slow dividing cells, have a very low transfection efficiency since most transfection methods require the cells to be in the $S/G_{(2)}/M$ phase to incorporate the plasmid into the genome of the cell. Lentiviruses have been shown to transduce both proliferating and non-proliferating cells, and allows for long term stable expression of the incorporated transgene[302]. Improved safety features, such as self inactivating long terminal repeats (SIN-LTRs) and minimal split packaging designs[303], makes the SIN-lentiviruses the best candidate for the delivery and integration of short hairpin RNA (shRNA) against mlgd1 and 2 into HSCs.

Five shRNA sequences specific to the mlgd1 and mlgd2 were designed and cloned into lentiviruses along with an off-target shRNA sequence to be use as a control. To test which shRNA sequence most efficiently knocks down the target genes and proteins, the murine fibroblastic cell line NIH-3T3 was infected at a multiplicity of infection (MOI) of 25 with lentivirus expressing shRNA sequences against mlgd1, mlgd2 and the off-target for


A)
7 days incubation
ShRNA against mLgd1
1
2
3
4
5
Off-target
150
100
75
50
35
Anti-mLgd1
Anti-actin


B)
10 days incubation
ShRNA against mLgd1
1
2
3
4
5
Off-target
150
100
75
50
35
Anti-mLgd1
Anti-actin

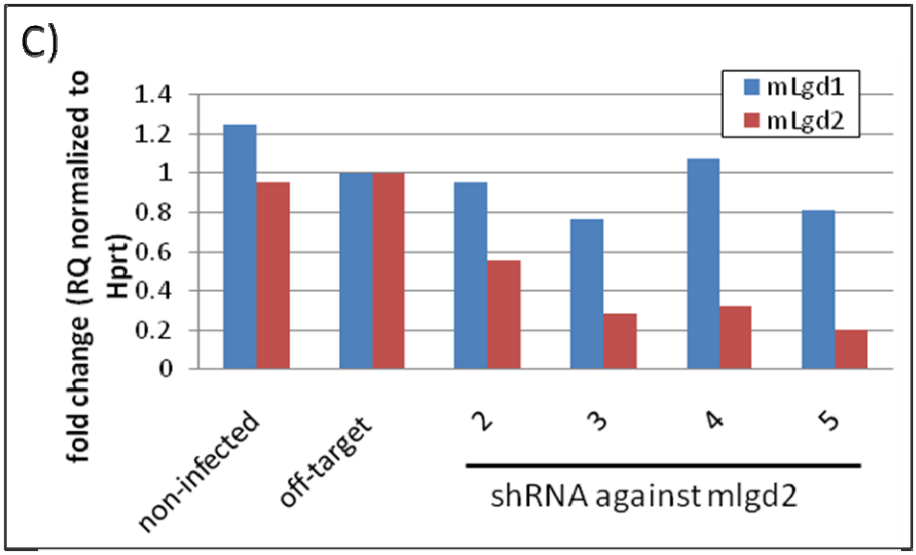

C)
fold change (RQ normalized to Hprt)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
mLgd1
mLgd2
non-infected
off-target
2
3
4
5
shRNA against mlgd2

Figure 10: Down regulation of the mLgd1 protein and the mlgd2 mRNA after stable expression of ShRNA against mldg1 and mlgd2 in NIH-3T3 cells. NIH-3T3 cells were infected (MOI 25) with lentivirus expressing shRNA against mlgd1, mlgd2 and an off-target sequence. Cells were treated for 4 days with puromycin (1µg/mL) to select cells expressing the shRNAs. Protein and mRNA was extracted after 7 and 10 days of the initial infection. Western blot of 50µg of total protein extracts isolated after 7 days (A) and 10 days (B) of incubation were probed with anti-hlgd1 and anti-actin. There appears to be a 30% decrease in the mlgd1 protein after 10 days of incubation. C) qPCR for the mlgd2 gene from total RNA extracted after 10 days of incubation. shRNA 3, 4 and 5 against mlgd2 decrease the expression of the mlgd2 gene by 70-80%. The shRNA4 appear to be the best candidate because it affects the mlgd2 gene only.

36 hours. Cells were then challenged with puromycine (1 μg/ml) for 4 days to selected cells that incorporated that the shRNA vectors. RNA and proteins were extracted at 7 and 10 days after initial infection.

Protein levels of mLgd1 were determined by western blot using a commercially available antibody against hLgd1 (Figure 10.A. & B.). There were no commercially available Lgd anti-bodies that specifically recognized the murine isoforms. A commercially available antibody that against hLgd2 was also tested however, it did not detect the murine form of mLgd2. Though mLgd1 is predicted to have a mass of 104 KDa, the anti-hLgd1 can detect the murine mLgd1 as either a single band or double band at approximately 125 KDa (Figure 10.A. & B.). This difference between the predicted molecular mass and the one observed is also seen when the antibody is used to detect the human hLgd1 isoform[42, 295]. Since very little is known on how the Lgd protein functions and how they are activated, it is possible that this difference in size could be due to post-translational modification such as phosphorylation, glycosylation, etc. Preliminary experiments suggest that a minimum of 10 days is required after initial lentiviral infection to observe a decrease in mLgd1 protein levels by shRNAs (Figure 10.B.). By calculating pixel density and normalizing it to actin levels, there appears to be approximately 30% decrease in mlgd1 protein levels. However, due to unequal protein loading and saturation of the actin signal, these results must be taken with caution and must be validated by further experiments.

To evaluate the effects of the shRNAs on the expression of the mlgd2 gene, qPCR analysis on extracted total RNA from NIH-3T3 cell after 10 days of the initial lentivirus infection was perform for the mlgd1 and mlgd2 genes (Figure 10.C.). mlgd1 and mlgd2 expression levels were normalized to three different control housekeeping genes: β-actin, hypoxanthine guanine phosphoribosyl transferase (Hprt) and TATA box binding protein (Tbp). The shRNA against mlgd1 and 2 appeared to have an effect on the expression of the β-actin gene, so it was not used as a control (data not shown). Though the data is not shown, similar results were obtained with the Tbp control gene as with Hprt control gene. The shRNA 3, 4 and 5 against *mlgd2* decrease the expression of the mlgd2 gene by 70-80% (Figure 10.C.). However, *mlgd2* shRNA4 appear to be the best candidate because it affected the mLgd2 gene only (Figure 10.C.). The effects of the *mlgd1* shRNAs on the expression of the mLgd1 gene were also evaluated by qPRC however, the results were

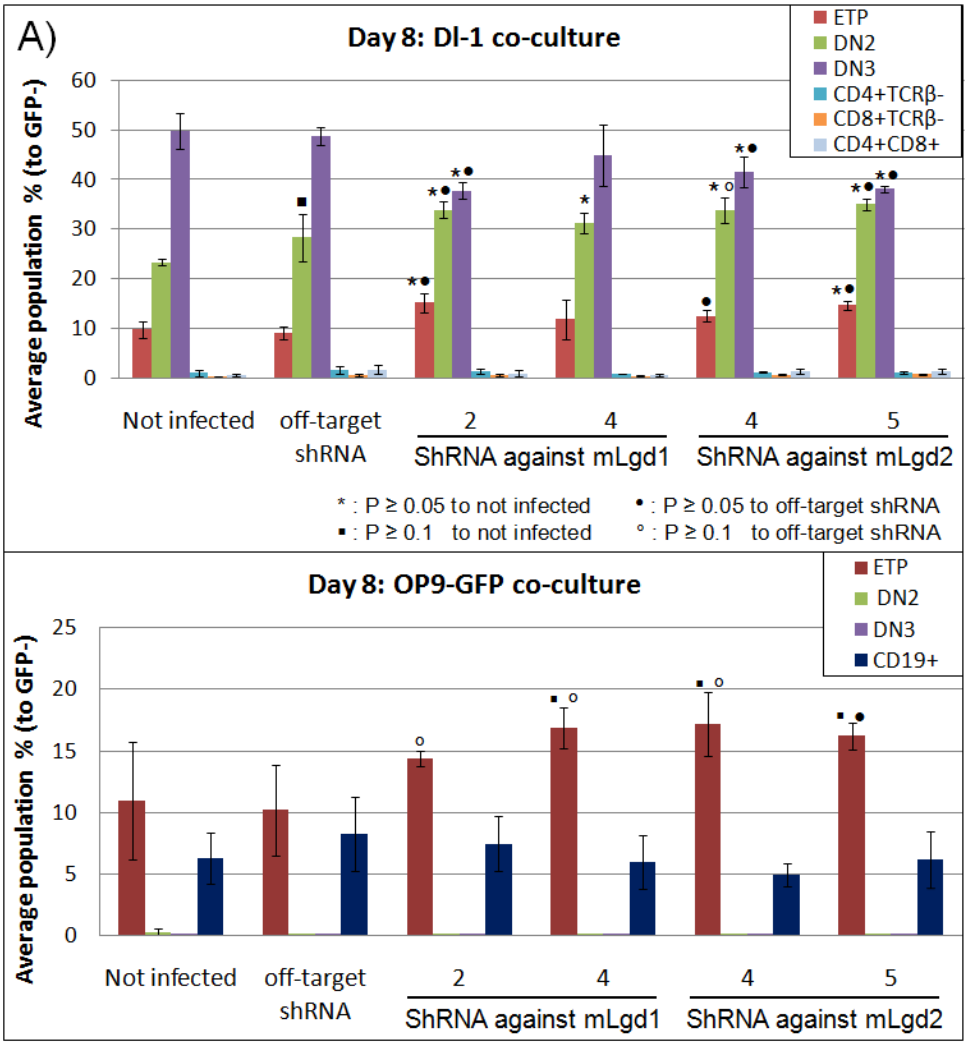
inconsistent. This inconsistency could be due to off target gene amplification due to inadequate primer selection or poor RNA quality.

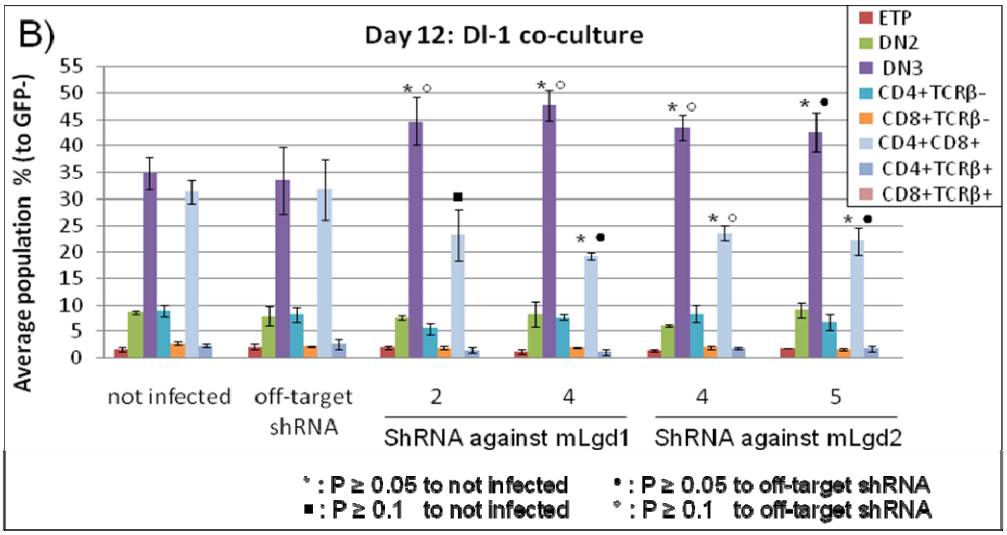
3.5. mlgd1 and mlgd2 modulates T-cell lineage development, most-likely due to Notch activation

T cell development can be studied *in vitro* using a two-dimensional cultures system where hematopoietic progenitors are exposed to Delta-like ligands that are expressed by cells[212-214]. In this study, the murine bone marrow stromal cell line, OP9, was used in a co-culture to support lymphoid lineage differentiation. E14.5 Foetal liver cells were harvested from pregnant C57BL/6 mice and used as a source of hematopoietic progenitors. Foetal liver cells were first expanded and prestimulated with cytokine for 24 hours and then infected for 48 hours with *mlgd1*, *mlgd2* or *off-target* shRNA lentiviruses (MOI 10). The foetal liver cells were co-cultured on top of OP9 cells transduced with the Notch ligand Delta-like-1 (OP9-Dl1) cells to supports T lymphopoiesis, as well as onto OP9 cells transduced with GFP (OP9-GFP) to supports B lymphopoiesis[215]. Cells were collected on day 8, 12 and 16 and FACS sorted to determine their stage of differentiation (Figure 11).

It is well established that overexpression of Notch1 in common lymphoid precursors drive T cell development at expense of natural killer, B lymphoid cells[206], and the myeloid lineage[304]. Under co-culture OP9-GFP B lymphopoiesis condition, cells expressing either the *mlgd1* or *mlgd2* shRNAs supported the maintenance of early T-cell progenitors (ETPs) compared to *off-target* control (Bottom panel, Figure 10.A.). Cells co-cultured on OP9-GFP were not collected on day 12 of co-culture due to a large number of dead cells observed when FACS analyzed on day 8. Cells were kept in culture and only sorted on day 16. Knocked down of the *mlgd1* and *mlgd2* genes had no overall affect on B cell development, which was the opposite of what was expected (Bottom panel, Figure 10.C.). This may be due to an inefficient overactivation of the Notch pathway.

After 8 days of co-culture, foetal liver cells expressing *mlgd1* shRNA 2 and *mlgd2* shRNAs 4 & 5 co-cultured under OP9-Dl1 T lymphopoiesis condition, also supported the maintenance and proliferation of early T-cell progenitors (ETPs) compared to *off-target* and


A)
Day 8: Dl-1 co-culture
ETP
DN2
DN3
CD4+TCRβ-
CD8+TCRβ-
CD4+CD8+
Average population % (to GFP-)
Not infected
off-target shRNA
2
4
4
5
ShRNA against mLgd1
ShRNA against mLgd2
* : P ≥ 0.05 to not infected
• : P ≥ 0.05 to off-target shRNA
▪ : P ≥ 0.1 to not infected
° : P ≥ 0.1 to off-target shRNA
Day 8: OP9-GFP co-culture
ETP
DN2
DN3
CD19+
Average population % (to GFP-)
Not infected
off-target shRNA
2
4
4
5
ShRNA against mLgd1
ShRNA against mLgd2
T-cell development
ETP
B Cell
DN2
DN3
TRCβ
CD4+ CD8+
CD4+
CD8+


B)
Day 12: Dl-1 co-culture
ETP
DN2
DN3
CD4+TCRβ-
CD8+TCRβ-
CD4+CD8+
CD4+TCRβ+
CD8+TCRβ+
Average population % (to GFP-)
not infected
off-target shRNA
2
4
4
5
ShRNA against mLgd1
ShRNA against mLgd2
* : P ≥ 0.05 to not infected
• : P ≥ 0.05 to off-target shRNA
▪ : P ≥ 0.1 to not infected
° : P ≥ 0.1 to off-target shRNA

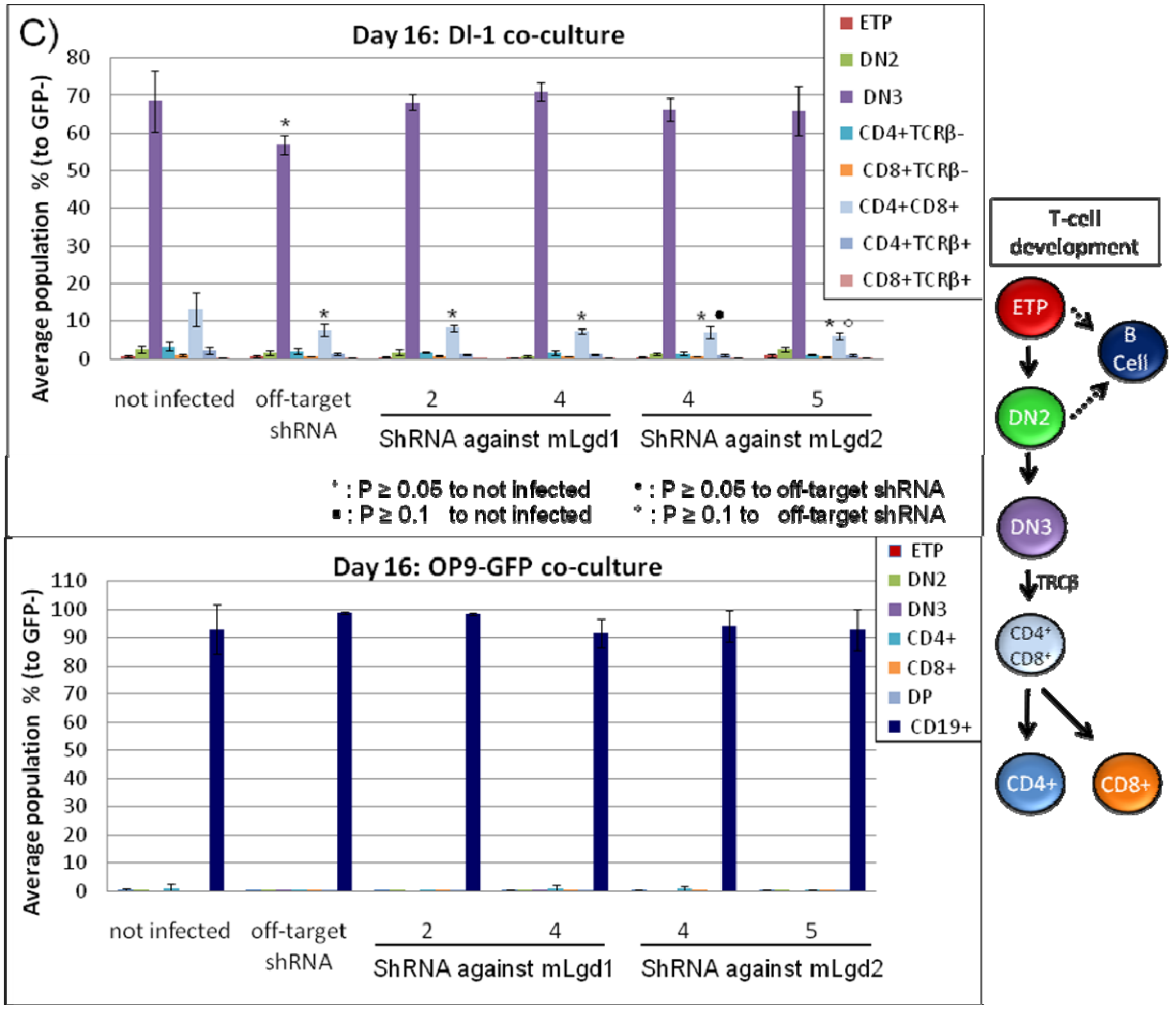


Figure 11: shRNA knockdown of mlgd1 and mlgd2 in foetal liver cells speeds up development of progenitors into the T-cell lineage, but causes a block at DN3 stage. E14.5 Foetal liver cells were harvested from pregnant C57BL/6 mice, prestimulated for 24 hr and then infected for 48 hr with mLgd1, mLgd2 or off-target shRNA lentiviruses (MOI 10). Cells were then plated onto OP9-GFP expressing cells to stimulate B-cell differentiation or onto OP9-Dl1 to stimulate T-cells differentiation. Cells were collected on day 8 (A), 12 (B) and 16 (C) and FACS sorted to determine their stage of differentiation. Knockdown of mLgd1 and 2 did not have any effect on B-cell differentiation (A and C bottom panels). Knockdown of mLgd1 and 2 accelerated foetal liver cell differentiation into the T-cell lineage (A top panel, B), however these cells were blocked at the DN3 stage (C, top panel). A student's T-test was performed on the data collected. ETP: early T-cell progenitor, DN: double negative, SP: single positive, DP: double positive

non-treated controls (Top panel, Figure 11.A.). Cells expressing the *mlgd1* and *2* shRNAs had a significant larger DN2 population levels compared non-treated control. A significant higher DN2 population level was also observed for cells expressing *mlgd1* shRNA 2 and *mlgd2* shRNAs 4 & 5 compared to the *off-target* shRNA control (Top panel, Figure 11.A.). However, these cells had a significantly lower DN3 population compared to *off-target* and non-treated controls as well. By day 12 of OP9-Dl1 co-culture, the DN3 population was significantly higher and the $CD4^{+}CD8^{+}$ double positive (DP) population level was significantly lower in cells expressing the *mlgd1* and *mlgd2* shRNAs compared to both the *off-target* shRNA and non-treated control cells (Figure 11.B.). This indicates that down regulation of *mlgd1* and *mlgd2* causes an accelerated differentiation of hematopoietic stem cell into the T lymphopoiesis lineage. However at 16 of OP9-Dl1 co-culture, the DN3 population level of the non-treated cells was similar to those of the cells expressing the *mlgd1* and *mlgd2* shRNAs (Top panel, Figure 11.C.). Though one would expect the $CD4^{+}CD8^{+}$ DP population levels of the cells expressing the *mlgd1* and *mlgd2* shRNAs to be greater than those of the non-treated cells, they are actually significantly lower (Top panel, Figure 11.C.). This suggests that downregulation of *mlgd1* and *mlgd2* causes a block in the DN3 to $CD4^{+}CD8^{+}$ DP transition. The overall different populations' levels of the cells expressing the *off-target* shRNA were significantly lower than the other groups (Top panel, Figure 11.C.). When the sample was FACS sorted, it had the lowest total population events collected and appeared to have higher incidence of cellular death compared to the other groups. An error in experimental manipulation of the *off-target* shRNA expressing cells on day 16 of OP9-Dl1 co-culture most-likely occured, thus it was not used for comparison with the other groups. Overall the fact that down regulation of *mlgd1* and *mlgd2* causes an accelerated differentiation of hematopoietic stem cell into the T lymphopoiesis lineage and that there was a block in block in the DN3 to $CD4^{+}CD8^{+}$ DP transition, suggests that Notch is overactivated in these cells[203, 232].

Discussion

Notch signalling is essential to many developmental processes and has been shown to regulate stem cell self-renewal, cell proliferation, cell differentiation and apoptosis. Loss of function of various components of the Notch signalling pathway is known to be the cause of some inherited diseases[13] and different types of cancers[14, 15]. Thanks to studies performed in the *Drosophila* model system, it was revealed that endocytosis plays a critical role in the regulation of Notch activation and processing of its ligands.

The *Drosophila* gene lethal giant discs (dlgd), named after its loss of function phenotype, has been shown to play a role in the regulation of the Notch signalling. Mutations in this gene cause overgrowth of the imaginal discs through a perturbation in Notch endocytosis[37-40]. Though dLgd has been characterized to function in the endocytic pathway, its precise function is unknown. Even less is known of the mammalian lgd1 and 2. In a *Drosophila* loss-of-function experiment, both the murine *mlgd1* and *2* orthologs were able to rescue the *dlgd* loss-of-function phenotype, thus demonstrating a conservation of function between homologs[39]. However, recent publications suggest that they may act differently in mammals than in *Drosophila*[41-44]. In mammals, published data shows or strongly suggest that the Lgd homologs are not endocytic proteins but nuclear[41, 43, 44].

Notch signalling is well known to play a role in regulating the maintenance of neuronal stem cells and cell fate choice in the central nervous system of both *Drosophila* and mammals. In *Drosophila* experiments, loss-of-function *Notch* causes neuroblast to differentiate into neural cells[259, 260], while overactivation of Notch pathway causes overperliferation and loss of differentiation of neuroblasts [266]. Recent studies have implicated the two human *Drosophila* lgd orthologs, hlgd1 and hlgd2, in brain development as well [295, 296]. This was why the *Drosophila* neuroblast was chosen as model system in the first part of this study to validate that dLgd is implicated in Notch signalling. We demonstrated that $dlgd^{08}$ mutants induced a dramatic overproliferation of neuroblasts (Figure 7.A.) and an accumulation of the Notch receptor in vesicular structures (Figure 8) compared to wild-type (WT). This was similar to the $rab5^{QL}$ mutants (Figure 7.A.) which have been shown to cause an accumulation of the Notch receptor in the early endosome, overactivation of the Notch signalling pathway and overproliferation of cells in the wing disc[299]. The $dlgd^{08}$ mutant *Drosophila* phenotype appears to be caused by an

overactivation of Notch due to its accumulation in vesicles, suggests that Notch is activated from within endosomes in the *Drosophila* neuroblasts. Taking into account these observations, and that Notch is implicated neuronal differentiation [270] in mammals, indicates that mutations of hLgd in NSMR patients may also cause an accumulation of the Notch receptor in the endosome and overactivation of the Notch pathway in the patient's neuronal stem cells.

Very little is known about the mammalian *lgd1* and *2*. It has been shown that both the murine *mlgd1* and *2* are able to rescue the *dlgd* loss-of-function Notch overactivation and wing disc overproliferation phenotypes in *Drosophila*, thus demonstrating a conservation of function between homologs[39]. Studies performed in the murine, rat and human brain or neuronal cells have shown that the mammalian lgd1 and 2 homologs function as a transcription regulator for 5-HT1A serotonin receptor and are located in the nucleus[41, 43, 44, 298, 305]. Thus it was of interest to determine whether the human orthologs hlgd1 and 2 were located in the endocytic compartment or in the nucleus. Immunohistochemistry was performed on the human cervical immortal cell line cells, Hela. hLgd-1 and 2 were found in distinctive punctea located in the cytoplasm (Figure 9.A.), when cells stained with both anti-hLgd1 and anti-hLdg2, and not in the nucleus as previously observed[41, 298]. Though there is some co-localization between all three, the endosome, hlgd1 and 2, it appears that hlgd1 and 2 are localized in different compartments (Figure 9.A.). Co-localization studies with hLdg2 and the early endosome marker EEA1 (Figure 9.B) or the late endosome LAMP2 (Figure 9.C.) suggest that hLgd2 is associated with the late endosome and lysosome while hLgd1 is associated with the early endosome. However further investigation is required to pinpoint the exact locations of the hLgd proteins in the cytoplasm. Studies in *Drosophila* have demonstrated that dLgd is located in the endocytic pathway after presenilin and hrs, but before the ESCRT complex[37, 39, 40]. Thus, it would be of interest to continue the immunohistochemistry and compare the location of dLgd, hLgd1 and hLgd2 to a panel of known endocytic proteins.

The next aim of the study was to determine if mammalian lgd1 and 2 also regulate Notch signaling in mammals. One of the most studied Notch dependent system is the differentiation of the T cell lineage. Thus to best study the effects of mammalian lgd, it was decided to knockdown the murine *mlgd1* and *2* in hematopoietic stem cells (HSCs) and

observe how it affects T cell differentiation using an *ex vivo* T cell differentiation assay[215]. To test which shRNA sequence most efficiently knocks down the target genes and proteins, NIH-3T3 cells were infected with the various lentiviruses. Total protein and RNA were extracted from these cells and the mLgd 1 and 2 protein and RNA levels were determined by western blot and qPRC, respectively (Figure 10).

Protein levels of mLgd1 and 2 were detected using commercially available antibodies against the hLgds, since there were no murine mLgd1 and 2 antibodies available. The antibody against hLgd2 did not detect the murine form of mLgd2. Preliminary experiments suggest that a minimum of 10 days is required after initial lentiviral infection to observe a decrease in mLgd1 protein levels due to shRNA (Figure 10.B.). By calculating pixel density and normalizing it to actin levels, there appears to be approximately 30% decrease in mlgd1 protein levels. However, due to unequal protein loading and saturation of the actin signal, these results must be taken with caution and must be validated by further experiments. It would be of interest to create tagged mlgd1 and 2 clones in order to better identify these protein in either immunohistochemistry experiments or by western blots since there are no a commercially available murine antibodies for the mLgd proteins. It would also be advisable to create homemade mLgd1 and mLgd2 antibodies.

The RNA levels were evaluated for the *mlgd1* and *mlgd2* genes by qPCR (Figure 10.C.). The *mlgd2* shRNA 3, 4 and 5 decrease the expression of the *mlgd2* gene by 70-80% (Figure 10.C.). However, *mlgd2* shRNA4 appear to be the best candidate because it affects the *mlgd2* gene only (Figure 10.C.). The effects of the *mlgd1* shRNAs on the expression of the *mlgd1* gene were also evaluated by qPRC however, the results were inconsistent. This inconsistency could be due to off target gene amplification due to inadequate primer selection or poor RNA quality. New primers need to be designed and RNA quality should be check prior to reversed transcription.

T cell development was studied using the *ex vitro* OP9-Dl1 two-dimensional cultures system[212-214]. E14.5 Foetal liver cells were harvested from pregnant C57BL/6 mice and used as a source of hematopoietic progenitors. Foetal liver cells were first expended and pre stimulated with cytokine for 24 hours and then infected for 48 hr with *mlgd1*, *mlgd2* or control shRNA lentiviruses. Cytokine stimulation of the quiescent hematopoietic progenitors prior to lentiviral transduction induces cell cycling which is believed to

improve the transduction efficiency of reconstituting stem cells and help induce differentiation of HSCs[306, 307].

When foetal liver cells were co-culture onto OP9-GFP, favouring B lymphopoiesis, downregulation of both *mlgd1* and *mlgd2* by shRNAs supported the maintenance of early T-cell progenitors (ETPs) compared to *off-target* control (Bottom panel, Figure 10.A.). Notch signalling is known to be essential for the generation of foetal hematopoietic stem cells (HSCs) [210, 211], thus this suggests that down regulation of *mlgd1* and *2* may be activating the Notch pathway. However the *mlgd1* and *mlgd2* shRNAs had no overall affect on B cell development, which was the opposite of what was expected (Bottom panel, Figure 10.C.). This may be due to inefficient overactivation of the Notch pathway.

Downregulation of both *mlgd1* and *mlgd2* also induced proliferation and help maintain ETP population of foetal liver cells co-culture onto OP9-Dll1, favouring T lymphopoiesis, compared to off-target and non-treated controls (Top panel, Figure 11.A.) In addition, it also caused an accelerated differentiation of hematopoietic stem cell into the T lymphopoiesis lineage. There was a greater population of DN2 cells at day 8 of co-culture (Top panel, Figure 11.A.) and of DN3 population at day 12 (Figure 11.B) compared to *off-target* shRNA and non-treated control. The exact role Notch signalling in ETP-DN2 transition is ambiguous, however Notch signalling is required for the maintenance of CD25 expression in DN2 and DN3 cells and the survival of DN2, DN3 and DN4 cells[220, 221]. Thus overactivation of the Notch signalling could explain this accelerated differentiation.

At 16 days of OP9-Dl1 co-culture, the DN3 population level of the non-treated cells is similar to those of the cells expressing the *mlgd1* and *mlgd2* shRNAs (Top panel, Figure 11.C.). However, one would expect that the $CD4^+CD8^+$ DP population levels of the *mlgd1* and *mlgd2* shRNAs expressing cells would be greater than those of the non-treated cells since the DN3 levels were much greater at day 12. This suggests that downregulation of *mlgd1* and *mlgd2* causes a block in the DN3 to $CD4^+CD8^+$ DP transition. This sort of phenotype is also observe when Presenilin1/2 is downregulated[231]. Notch signalling is maintained in mice until the β selection or pre-TCR checkpoint and is rapidly downregulated afterwards[222, 223]. Deletion of *Presenilin1* is initiated at the DN2-DN3 stage, thus occurs prior to TCRβ selection[231]. This severely impacts DP thymocyte number because the cells cannot mature past the DN3 stage[203, 232]. The deletion of

genes that impact Notch signalling, which is induced after the DP stage or TCRβ selection, such as deletion of the CSL gene and mastermind, generate normal numbers of DP thymocytes[229, 230], indicating that Notch signalling is down regulated later, most likely after TCRβ selection. This suggests that in the case of *mlgd1* and *mlgd2* downregulation, Notch is overactivated in the thymocytes prior to pre-TCRβ selection. However, it cannot be concluded that deregulation of Notch signalling is the sole reason why downregulation of *mlgd1* and *mlgd2* causes an accelerated T cell differentiation in the foetal liver cells. In *Drosophila* mutations of *dlgd* causes an accumulation of Notch in enlarges early endosome punctas, ubiquitinated protein and the EGF receptor as well, indicating that *dlgd* is required for general protein sorting[38, 39]. This suggests that deregulation of *mlgd1* and *2* in HSCs may trigger general defects in endocytosis and thus cause perturbation in other signalling pathways important in the T cell differentiation process. Further investigation is required to validate that downregulation of *mlgd1* and *mlgd2* causes accelerated T cell differentiation in the foetal liver cells is due to Notch overactivation. The T cell differentiation assay needs to be redone with proper Notch controls such as, over and down expression of the Notch receptor and target genes. Also over and down expression of known endocytic protein that upregulate Notch signalling or have no affects.

Currently, to further analyze the role of mammalian Lgd homologs, ES-cells knock-out for mLgd1 and 2 were ordered from the KOMP consortium in order to generate conditional knockout (KO) mice. If the null mice are lethal, various CRE lines will be used to generate tissues-specific nulls, such as the CRE lines Nestin-Cre[308] for the brain and Lck-Cre[309] and Vav-Cre[310] for the immune system. Initial analysis of the morphology of null mice for either one or the combination of both mLgd will first focus on brain development since Notch plays a critical role in brain development and the implications of hLgd1 and 2 in mental retardation[295, 296]. Then the components of the peripheral blood, such as the proportion of T- and B-cell, will be examined since Notch signalling is essential for T-cell differentiation[206].

From the generated Lgd null mouse, mouse embryonic fibroblasts (MEFs) cell lines for mLgd1$^{-/-}$ MEFs, mLgl2$^{-/-}$ MEFs and double KO MEFs would also be established in order to compare endosomal morphology and function to that of wild type MEFs.

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Appendices

Université de Montréal

Service de l'admission et du recrutement

Le 11 février 2010

Votre code permanent: [redacted]

Andréa Hébert-Losier
[redacted]

(non résidente du Québec)

Admission

Madame,

J'ai le plaisir de vous informer que vous êtes admise à:

2-466-1-0 M.Sc. (Biologie moléculaire)

pour l'hiver 2010 comme étudiante régulière à plein temps.

Je vous souhaite la bienvenue et du succès dans vos études.

. durée minimale de la scolarité (en trimestres équivalent plein temps): 3.0 trimestre(s).
durée de la scolarité (en trimestres équivalent plein temps) accordée en exemption: 3.0 trimestre(s).

. N.B. : 3 crédits transférés pour le moment

N.B. Vous êtes autorisée à soumettre un mémoire rédigé en langue anglaise.

Responsable : Jocelyne Emond tél : 514 343-6426
pour Louise Béliveau, Doyenne
Études supérieures

[redacted]

José Bourguignon, Directrice