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Communauté UNIVERSITÉ Grenoble Alpes

THÈSE

Pour obtenir le grade de

DOCTEUR DE LA COMMUNAUTE UNIVERSITE GRENOBLE ALPES

Spécialité : **Biologie Cellulaire**

Arrêté ministériel : 25 mai 2016

Présentée par

Xènia CRESPO-YAÑEZ

Thèse dirigée par **Marie-Odile FAUVARQUE**

préparée au sein du **Laboratoire Biologie à Grande Echelle, Institut de Biosciences et Biotechnologies de Grenoble, CEA Grenoble**
dans **l'École Doctorale de Chimie et Sciences du Vivant**

Découverte de l'ubiquitination en tant que nouveau mécanisme de régulation de la protéine ESCRT-III CHMP1B

Thèse soutenue publiquement le **13 octobre 2017**
devant le jury composé de :

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Synopsis en français

J'ai effectué ma thèse dans le groupe du Dr. Marie-Odile Fauvarque qui met en œuvre des stratégies de génétique moléculaire sur des modèles de cellules humaines en culture et chez la mouche drosophile pour l'identification et l'étude de la fonction des protéines dans la signalisation intracellulaire. Dans ce contexte, mes travaux visaient à produire des connaissances fondamentales sur le système ubiquitine dans le contrôle du trafic endocytaire, en particulier de récepteurs membranaires impliqués dans la réponse inflammatoire (TNFR, ILR) ou la différenciation et la croissance cellulaire (EGFR). Je me suis notamment intéressée au rôle du complexe formé par l'interaction entre une protéine de la voie endocytaire, CHMP1B, et la protéase d'ubiquitine UBPY (synonyme USP8). CHMP1B est un membre de la famille ESCRT-III qui, *via* des processus de changements de conformation et de polymérisation à la membrane, contrôle la biogenèse des vésicules intraluménales (ILVs) au niveau des endosomes tardifs pour former les corps multivésiculaires (MVBs). Ces derniers fusionnent avec les lysosomes, assurant ainsi la protéolyse des récepteurs internalisés et l'arrêt de la signalisation intracellulaire. Alternativement, les récepteurs peuvent être renvoyés à la membrane plasmique à partir des endosomes précoces ou tardifs *via* des vésicules de recyclage. Le trafic intracellulaire et le tri des récepteurs dans ces différents compartiments subcellulaires jouent un rôle majeur dans l'activation, la durée et la terminaison des signaux intracellulaires. Or, la liaison covalente d'une ou plusieurs ubiquitine (un polypeptide très conservé de 76 aminoacides) au niveau des récepteurs est un signal majeur déclenchant leur internalisation. En hydrolysant cette ubiquitine, UBPY peut stopper l'internalisation des récepteurs au niveau de la membrane plasmique, ou bien, favoriser leur entrée dans le MVB. UBPY jouerait ainsi deux rôles opposés sur la stabilité des récepteurs selon son niveau d'action dans la cellule. L'interaction entre les deux protéines CHMP1B et UBPY avait été décrite dans la littérature dans le système deux-hybrides chez la levure ou par co-immunoprécipitation à partir de lysat cellulaires. Cependant, les travaux de l'équipe montraient l'absence d'interaction forte entre les domaines d'interaction de ces deux protéines *in vitro* et par ailleurs, la fonction de cette interaction dans le processus d'endocytose n'avait été que partiellement élucidée.

Au cours de ma thèse, j'ai confirmé l'existence du complexe CHMP1B-UBPY *in cellulo* qui se localise essentiellement au niveau des endosomes tardifs. J'ai déterminé la région impliquée dans cette interaction et prouvé que l'existence de ce complexe permet de stabiliser les deux protéines dans les cellules. J'ai ensuite démontré l'existence de formes ubiquitinées monomériques et dimériques de CHMP1B dans lesquelles la liaison d'une molécule d'ubiquitine sur une des deux lysines d'une boucle flexible de la protéine induit un probable changement de conformation. De plus, UBPY hydrolyse cette ubiquitine et favorise l'accumulation d'oligomères de CHMP1B qui sont dépourvues d'ubiquitine. Finalement, le traitement des cellules par l'EGF, qui se lie à l'EGFR et provoque son internalisation, induit le recrutement transitoire des dimères ubiquitinés de CHMP1B aux membranes. L'analyse du trafic intracellulaire de l'EGFR et de la morphogenèse de l'aile de drosophile dans différents contextes génétiques a également prouvé que la forme ubiquitinée de CHMP1B est essentielle à sa fonction. L'ensemble de mes travaux m'autorisent à formuler une

hypothèse complètement nouvelle dans laquelle l'ubiquitination de CHMP1B induit une conformation ouverte de la protéine incapable de polymériser qui est recrutée sous forme de dimères à la membrane des endosomes où la présence d'UBPY induit la deubiquitination et la polymérisation concomitante de CHMP1B, très probablement en hétéro-complexes avec d'autres membres de la famille ESCRT-III agissant de concert pour la déformation et la scission des membranes.

Synopsis in English

I did my thesis in the group of Dr. Marie-Odile Fauvarque who implements strategies of molecular genetics on human cell culture models and in the *Drosophila* fly for the identification and study of the function of proteins in intracellular signaling. In this context, my work aimed to produce fundamental knowledge about the ubiquitin system in the control of the endocytic trafficking, in particular of membrane receptors involved in the inflammatory response (TNFR, ILR) or cell differentiation and growth (EGFR). I was particularly interested in the role of the complex formed by the interaction between an endocytic protein, CHMP1B, and the ubiquitin protease UBPY (synonym USP8). CHMP1B is a member of the ESCRT-III family that controls the biogenesis of intraluminal vesicles (ILVs) at the late endosomes to form multivesicular bodies (MVBs) Conformational change and polymerization at lipidic membrane processes are needed for CHMP1B function. MVBs fuse with the lysosomes, thus ensuring the proteolysis of the internalized receptors and the stoppage of the intracellular signaling. Alternatively, the receptors may be returned to the plasma membrane from early or late endosomes via recycling vesicles. Intracellular trafficking and receptor sorting in these different subcellular compartments play a major role in the activation, duration and termination of intracellular signals. The covalent bond of one or more ubiquitin (a highly conserved polypeptide of 76 amino acids) at the receptors is a major signal triggering their internalization. By hydrolyzing this ubiquitin, UBPY can stop the internalization of receptors at the plasma membrane, or promote their entry into the MVB. UBPY would thus play two opposing roles on the stability of the receptors depending on its level of action in the cell. The interaction between the two proteins CHMP1B and UBPY had been described in the literature in the two-hybrid system in yeast or by co-immunoprecipitation from cell lysates. However, the team's work showed no strong interaction between the domains of interaction of these two proteins *in vitro* and the function of this interaction in the endocytosis process had only been partially elucidated.

During my thesis, I confirmed the existence of the CHMP1B-UBPY *in cellulo* complex, which is located mainly at the level of late endosomes. I determined the region involved in this interaction and proved that the existence of this complex makes possible the stabilization of both proteins into the cells. I then demonstrated the existence of monomeric and dimeric ubiquitinated forms of CHMP1B in which the binding of a molecule of ubiquitin to one of the two lysines of a flexible loop of the protein likely induces and/or stabilize a

conformational conformation. In addition, UBPY hydrolyses this ubiquitin and promotes the accumulation of CHMP1B oligomers which are devoid of ubiquitin. Finally, the treatment of cells by EGF, which binds to EGFR and causes its internalization, induces transient recruitment of ubiquitinated CHMP1B dimers to the membranes. Analysis of the intracellular trafficking of EGFR and the morphogenesis of *Drosophila* wing in different genetic contexts has also shown that the ubiquitination of CHMP1B is essential to its function. My work has allowed me to formulate a completely new hypothesis in which the ubiquitination of CHMP1B induces an open conformation of the protein incapable of polymerizing in this state which is recruited in the form of dimers to the membrane of the endosomes and there the presence of UBPY induces the deubiquitination and the concomitant polymerization of CHMP1B, most probably in hetero-complexes with other members of the ESCRT-III family acting in concert for deformation and scission of the membranes.

This work has been submitted for publication and a number of revisions have been made according to reviewer's remarks.
Xènia CRESPO-YÀÑEZ, Carmen AGUILAR-GURRIERI, Anne-Claire JACOMIN, Emmanuelle SOLEILHAC, Emmanuel TAILLEBOURG, Winfried WEISSENHORN and Marie-Odile FAUVARQUE.
Unveiling ubiquitination as a new regulatory mechanism of ESCRT protein CHMP1B, *Submitted*
In addition, I will be part of the authors of other work performed in collaboration with members of the laboratory.

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Abbreviations and Acronyms

A

aa: amino acid
ADAM: a Disintegrin and metalloproteinase
AIP4/ITCH: Atrophin-1-interacting protein 4
Alix: ALG-2-interacting protein X
Ap: Apterous
AMSH: Associated molecule with SH3 domain of STAM
ap: Apterous
AP2: Adaptin protein 2
Arf6: ADP-ribosylation factor 6
Atg: Autophagy-related genes
ATP: Adenosine triphosphate

C

CARD: Caspase activation and recruitment domain
CavME: Caveolae-mediated endocytosis
Cbl: Casitas B-linage lymphoma enzyme/ Cas-Br-M ecotropic retroviral transforming sequence
CCP: Clathrin-coated pits
CCV: Clathrin-coated vesicles
CEP55: Centrosomal protein 55
CHMP: Charged multivesicular bodies protein (or chromatin modifying protein)
CIE: Clathrin independent endocytosis
CIN85: (or SH3KBP1) SH3 domain-containing kinase-binding protein 1
CLIC: Clathrin independent carriers
CME: Clathrin mediated endocytosis
CYLD: Cylindromatosis

D

DC: Dendritic Cell
DNA: Deoxyribonucleic acid
DOK2: Docking protein 2
Dpp: Decapentaplegic
DUB: Deubiquitinating enzyme

E

EE: Early endosomes
EBV: Epstein-Barr virus
EEA1: Early endosomal antigen 1
EGF: Epidermal growth factor
EGFP: Enhanced green fluorescent protein
EGFR: Epidermal growth factor receptor
En: Engrailed
Eph: Ephrine
Eps15: Epidermal growth factor receptor substrate 15
ErbB: Erythroblastic leukemia viral oncogene
ERC: Endocytic recycling compartment

ERK: Extracellular signal–regulated kinases
ESCRT: Endosomal sorting complex required for transport

F

Fat10: HLA-F-adjacent transcript 10
FECA: First eukaryotic common ancestor
FEME: Endophilin-mediated endocytosis
FGF: Fibroblast growth factor
FLP: Flippase
Fng: Fringe
FRT: FLP recombinase target sequence
FYVE domain: Fab 1 (yeast orthologue of PIKfyve), YOTB, Vac1 (vesicle transport protein), and EEA1 domain
Fz-2: Frizzled2

G

Gag: Group-specific antigen
Gal4: Galactose-responsive transcription factor GAL4
GAP: GTPase-activating proteins
GDP: Guanosine diphosphate
GEEC: Glyco-phosphatidyl-inositol-anchored protein-enriched endosomal compartments
GEF: Guanine-nucleotide exchange factors
GFP: Green fluorescent protein
GPCR: G protein–coupled receptor
Grb2: Growth factor receptor-bound protein 2
GTP: Guanosine triphosphate
GUV: Giant unilamellar vesicles

H

HA: Human influenza hemagglutinin
HB: Heparin binding
HCS: High Content Screening
HECT: Homologous to the E6-AP carboxyl terminus
HeLa: Henrietta Lacks cells derived from a cervical carcinoma
HGF: Hepatocyte growth factor
HH: Hedgehog
HIV: Human immunodeficiency virus
HOPS: Homotypic fusion and vacuole protein sorting
Hrs: HGF-regulated tyrosine kinase substrate
HSC70: Heat-shock cognate 70
Hsp: Heat-shock promoter
Hub1: Homologue to ubiquitin 1

I

Ifm1: Gene for translation initiation factor IF-2 protein
IKKβ: IκB Kinase
IL-1β/2: Interleukine-1β or 2
ILV: Intra-lumnal vesicle
IMD: Immune deficiency
ISG15: Interferon-stimulated gene 15

Ist1: Increased sodium tolerance 1 homolog

J-K

Jak-STAT: Janus kinase (JAK) - signal transducer and activator of transcription (STAT)
JAMM: JAB1/PAD1/MNP-domain-containing metalloenzyme
JNK: c-Jun N-terminal kinase
KB: Keratin-forming tumor cell line HeLa
kDa: Kilo Daltons
ke : endocytic rate constant

L

LAMP1: Lysosomal associated membrane protein 1
LBPA: 2,2'-dioleoyl lysobiphosphatidic acid
LC-MS: liquid chromatography–mass spectrometry
LE: Late endosomes
LECA: Last eukaryotic common ancestor
LIP5: Lyst interacting protein 5
LUBAC: Linear ubiquitin assembly complex
LUV: Large unilamellar vesicles

M

MAPK: Mitogen-activated protein kinase
MHCII: Major histocompatibility complex-II
MIT: Microtubule interacting and transport
MIM: MIT interacting moieties
MINDY: Motif interacting with Ub-containing Novel DUB
Mono-Ub: monoubiquitin
MS: Mass spectrometry
MVB: Multivesicular bodies
Myr: Myristoylation

N

NEDD8/4: Neural Precursor Cell Expressed, Developmentally Down-Regulated 8/4
Neur: Neuralized
NF-κB: Nuclear factor kappaB
Nls: Nuclear localization signal
NPC: Nuclear pore complexes
Nrdp1: RNF41 - E3 ubiquitin-protein ligase
NRE: Notch responsive elements
NTD: Notch-terminal domain

O-P

OTU: Ovarian-tumour related ubiquitin Proteases
PDGF: Platelet-derived growth factor
PI3K: Phospshatidylinositol 3-kinase
PIP: Phosphatidylinositol phospholipid
PhoX: Phosphoinositide-binding structural domain
PKA: Protein kinase A
PKB(Akt): Protein kinase B

PKC: Protein kinase C
PLC: Phospholipase C
PM: Plasma Membrane
POSH: Plenty of SH3
PSAP: Prosaposin
PTB: Phosphor-tyrosine Binding
PTK: Protein tyrosine kinase
PTP: Protein tyrosine-specific phosphatases
PVR: Poliovirus receptor
pY: Phosphotyrosine

R

Rab: Ras related in brain
RFP: Red fluorescent protein
RING: Really interesting new gene
RNA: Ribonucleic acid
RNAi: RNA interference
RhoA: Ras homolog gene family, member A
RTK: Receptor tyrosine kinase

S

SH2 or 3: Src homology 2 or 3
Shc: SHC transforming protein 1
shRNA: Short-hairpin RNA
SNARE: Soluble N-ethylmaleimide-sensitive factor-attachment protein receptor
SNX: Sorting nexin family proteins
Src: Proto-oncogene tyrosine-protein kinase Src
Srk: S-Receptor-like serine/threonine-protein kinase
STAM: Signal-transducing adaptor molecule
SUMO: Small ubiquitin-like modifier
Syk: Spleen tyrosine kinase

T

Tal: Tsg101-associated ligase
Tbc1d3: TBC (Tre-2, Bub2p, and Cdc16p) domain family member 3
TGF: Transforming growth factor
TGN: Trans-Golgi network
TNF: Tumour necrosis factor
TRK: Tyrosine kinase protein
TSG101: Tumor susceptibility gene 101

U

UAS: Upstream activating sequences
Ub: Ubiquitin (in polyUb, monoUb and formulas)
UBD: Ubiquitin binding domain
UBE4B: Ubiquitin conjugation factor E4 B
Ubl5: Ubiquitin-like 5
UBPY: Ubiquitin specific protease Y (synonym USP8)
UCH: Ubiquitin C-terminal hydrolase

Ufm1: Ubiquitin-fold modifier 1
UIM: Ubiquitin interacting motif
ULD: Ubiquitin-like domain
ULK3: Unc-51 like kinase 3
ULP: Ubiquitin-like proteins
USP: Ubiquitin specific protease

V-W-Y

VEGF: Vascular endothelial growth factor
vg: vestigial
Vn: Vein
Vps: Vacuolar protein sorting
Wg: Wingless
WID: Wing imaginal disc
Wnt: Wint/Wingless family
Y2H: Yeast two-hybrid

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I. Introduction

1. UBIQUITIN SYSTEM

1.1 Introduction

Ubiquitination consists in the post-translational modification by the covalent attachment of ubiquitin moieties to the lysine residues of targeted proteins. This modification will change the protein function and destiny (Hochstrasser 2000).

Although the ubiquitin polypeptide (Ub) was discovered in 1975 by Gideon Goldstein (Goldstein et al 1975), the history of the ubiquitin research field started in the 1980s. The laboratories of Avram Hershko in Israel and the laboratory of Alexander Varshavsky in Cambridge, MA were investigating the intracellular proteolysis and they complementarily discovered the ubiquitin conjugation and ubiquitin-mediated proteolysis in cell extracts, including the identification of the E1, E2, and E3 enzymes (Hershko et al. 1980; Varshavsky 2006). They discovered how the cell can regulate the presence of a given protein by marking it with a label consisting of the polypeptide ubiquitin. Ubiquitinated proteins are then rapidly degraded in cellular "waste disposers" called proteasomes (Novelprice.org, A. Ciechanover 1998). Since then, the field has expanded a lot and actually it is known that the ubiquitin system is one of the main post-translational regulatory systems within the cell. It is also now well described that different types of ubiquitination determine whether a modified protein is degraded by the proteasome or a change of fate: the ubiquitinated protein may be recruited in multiprotein complexes to initiate signaling cascades, undergo endocytosis, etc.…Actually, the ubiquitin modifications are not solely a signal for proteasomal degradation but it generates structural diversity to control distinct biological processes (Sadowski et al. 2012; Swatek and Komander 2016).

However, experiments to demonstrate covalent modification with ubiquitin *in vivo* can be quite challenging due to the low steady-state levels of the ubiquitinated forms as a result of 26S proteasome degradation and/or of the action of highly active deubiquitinating enzymes (DUBs) that remove the ubiquitin units (Pickart and Cohen 2004). Several different methods to determine whether a particular protein is ubiquitinated have been developed including highly informative Mass spectrometry analyses (Ellison and Hochstrasser 1991; Hochstrasser 1991; Beers and Callis 1993; Treier et al. 1994; Laney and Hochstrasser 2002a; Laney and Hochstrasser 2002b;

Emmerich and Cohen 2015; Lee et al. 2016a; Hewings et al. 2017; Witting et al. 2017).

1.2 The Ubiquitin Polypeptide

Ubiquitin is a polypeptide of 76 amino acids and 8,5 kDa of molecular mass. It has a C-terminal tail signature of a di-glycine (GG) sequence and includes seven lysine residues. Four genes in the human genome produce ubiquitin linear chains: PS27A, UBB, UBC and UBA52 (Kimura and Tanaka 2010) which needs to be processed by DUBs into ubiquitin monomers (Komander et al. 2009).

Ubiquitin secondary structure is a β-grasp fold formed by a mixed β-sheet with five strands and seven reverse turns connected and stabilized by 2 α-helix (β β α β β α β). The ubiquitin contains a hydrophobic core. Three hydrophobic residues found on the α-helix and 11 of the 13 hydrophobic residues from the β-sheet are involved in constructing this hydrophobic core (Vijay-Kumar et al. 1987a; Vijay-Kumar et al. 1987b; Pickart and Eddins 2004; Pickart and Fushman 2004). There is a prominent hydrophobic surface patch centered on Ile44 which is commonly used for interaction with the ubiquitin-binding domains (UBDs) (Hurley et al. 2006) (Fig. 1).

Ubiquitin is found from the *archaea* to the highest eukaryotes and is expressed ubiquitously in pluricellular organisms. The ubiquitin protein is highly conserved with just three amino acids of difference between the yeast and the human proteins.

Ubiquitin can be modified itself by conjugation of ULPs (Ubiquitin like proteins) modifiers, or by small chemical modifications such as phosphorylation or acetylation. Ubiquitin can be acetylated on K, or phosphorylated on S, T or Y residues, and each modification has the potential to dramatically alter the signaling outcome expanding the ubiquitin code by adding structural complexity (Koyano et al. 2014; Wauer et al. 2015; Swatek and Komander 2016; Ohtake and Tsuchiya 2017). In addition, 'unanchored' ubiquitin or ubiquitin chains exist in the cells and may perform second-messenger-like functions (Wright et al. 2016). Rreviewed in (Swatek and Komander 2016).

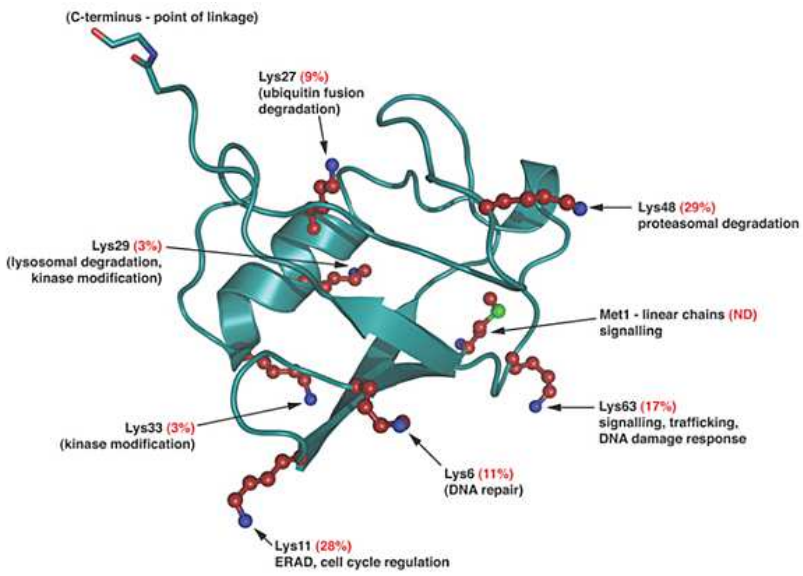


Figure 1: Ubiquitin structure: The structure of ubiquitin (green) reveals that all seven lysine residues (in red with nitrogen atoms in blue) reside on different parts at the surface of the molecule. Met1 (green sulphur atom) is the linkage point in linear chains and is close to K63. The C-terminal G75-G76 motif that forms the isopeptide bond is indicated (red oxygen atoms, blue nitrogen atoms). Lysine residues are labelled, and red numbers in parentheses refer to the relative abundance of the corresponding particular linkage in *S. cerevisiae* described in the proteomic study with His-tagged ubiquitin (Xu et al. 2009) (Modified from Komander 2009).

1.3 Ubiquitin-Like Proteins (ULPs) and Ubiquitin-Like Domains (ULDs)

1.3.1 ULPs

There are 17 ULPs found in mammalian cells. Indeed, the process of evolution has generated families of eukaryotic proteins that share with ubiquitin the β-grasp fold characteristic of Ub. Ubiquitin and ULPs proteins regulate many if not all cellular processes like nuclear transport, proteolysis, translation, autophagy, and antiviral pathways (Hochstrasser 2009; Heride et al. 2014; Li et al. 2016; Dikic 2017; Frankel and Audhya 2017; Gilberto and Peter 2017; Wang et al. 2017; Werner et al. 2017). These proteins share a common biochemical bound mechanism to target protein with the ubiquitin; however the function of ULPs can differ greatly from that of the ubiquitin (van der Veen and Ploegh 2012; Cappadocia and Lima 2017).

The main ULPs families are SUMO (small ubiquitin-like modifier) and NEDD8 (Neural Precursor Cell Expressed, Developmentally Down-Regulated 8). Beside, one can cite the linear di-ubiquitin analogues Fat10 and ISG15, and the single-domain protein Ufm1 as well as the Atg7, 8 and 12 (autophagy-related genes). ATG proteins are larger than the ubiquitin and do not share its structure; however they are considered as ULPs because they are mechanistically related to the Ub. Ufm1 and the newly discover Hub1/Ubl5 family are found only in mammals while the rest of the families are distributed among all the eukaryotes (Hu and Hochstrasser 2016).

SUMO Proteins

SUMO proteins are polypeptide of around 100 amino acids in length and 12 kDa in mass. They have a nearly identical structural fold as ubiquitin. The structure of human SUMO1 is globular with both ends of the amino acid chain branching out of the protein. The spherical core consists of an alpha helix and a beta sheet. In the SUMO system, SUMOylases are more abundant and diverse than E3s SUMO ligases. To date, the main known roles of SUMOylation are transcriptional regulation, control of protein stability, nuclear-cytosolic transport, regulation of genomic integrity and mitotic chromosome structure, regulation of recruitment of proteins to the kinetochore; also, many viral proteins have been shown to be modified by SUMO (Matunis et al. 1996; Johnson 2004; Hay 2005; Liebelt and Vertegaal 2016; Enserink 2017).

NEDD8 Proteins

NEDD8 is a polypeptide of 81 amino acids in length and around 9kDa in mass. The structure of human NEDD8 is very similar to that of ubiquitin. NEDD8 undergoes a metabolism which parallels that of ubiquitin. The main known functions for NEDD8 linkage to proteins, described so far, are cell cycle control and embryogenesis. There are also evidences of cooperation between the ubiquitin and NEDD8 pathways to control protein function (Whitby et al. 1998; Xirodimas 2008).

1.3.2 ULDs

The ubiquitin-like β-grasp fold or so called Ubiquitin like domain (ULD) is a conserved feature within ubiquitin and ULPs that can be also present in other proteins. ULDs represent a versatile interaction module that has arisen more than

once during evolution. ULDs are defined as a region of 45-80 amino acids which strongly resembles ubiquitin in primary sequence as well as in 3D structure. When these domains are intramolecular, they lack the terminal di-glycine motif and cannot be processed or conjugated. The ULDs can also participate in the regulation of the enzymatic activity of a number of E3 ligases like Parkin, deubiquitinases like USP14 or other signaling transduction elements like IKKβ in the NF-kβ pathway (Grabbe and Dikic 2009).

1.4 Ubiquitination Reaction: E1, E2 and E3 Enzymes

The ubiquitination reaction consists in the covalent attachment of ubiquitin moieties to the ε-amino group of a lysine residue within a targeted proteins or to the N-terminus of a target protein. This linkage forms an iso-peptide bond between the terminal glycine of ubiquitin or UBL proteins and the amino group of the lysine or first methionine of the target protein. Reviewed in Zheng, 2017 #551)(Hochstrasser 2000; Pickart 2001; Dye and Schulman 2007) (Fig. 2).

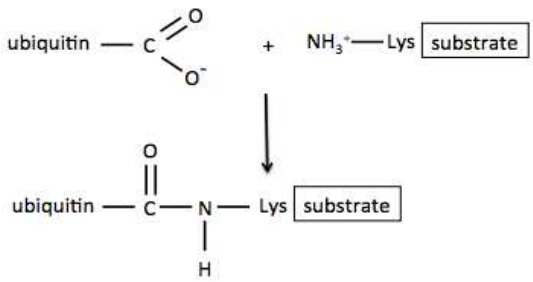


Figure 2: Representation of the isopeptide bond formation between an ubiquitin and a target protein

The conjugation of the ubiquitin to a protein involves three steps: an initial ATP-dependent activation step catalyzed by an E1 (activating enzyme), a second step in which the UBL is covalently linked to an E2 (conjugating enzyme), and a final step in which the UBL is covalently attached to its target protein amino group facilitated by an E3 (ligase) (Fig. 3).

The human genome encodes two E1 activating enzymes, 37 E2 conjugating enzymes and more than 600 E3 ligases. Nedd8 and Sumo each have a single E2 and a limited number of E3s. Atg8, Atg12, Ufm1, and ISG15 are each known to have a single E2-like enzyme, but so far, these UBLs lack known E3s. Neither E2s nor E3s are yet known in the cases of Urm1, Hub1, and Fat10.

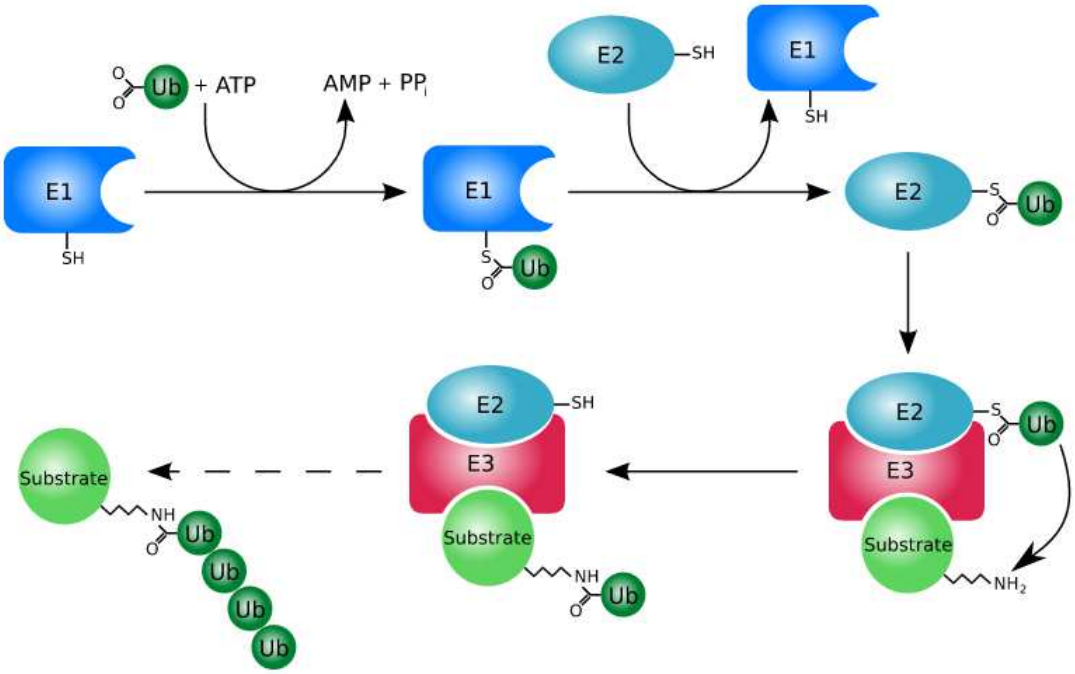


Figure 3: The ubiquitination pathway. An initial ATP-dependent activation step catalyzed by E1 (activating enzymes) is followed by a second step in which the ubiquitin is covalently linked to an E2 (conjugating enzyme), and a final step in which the ubiquitin is covalently attached to its target protein amino group facilitated by an E3 (ligase enzyme). (Image Created by Roger B. Dodd)

- **First step/E1 activating enzymes:** The first step activates the C-terminus of ubiquitin by generating a conformational change that will allow for attacking the substrate amino group. This reaction is ATP dependent and comprises two steps: an initial formation of an Ubiquitin-adenylate intermediate followed by the reaction of this intermediate with an E1 cysteine residue to form an E1~Ubiquitin thioester bound.
- **Second step/E2 conjugating enzymes:** In a second step, the ubiquitin is transferred from E1 to E2. The E2-ubiquitin interface includes the C-terminus of ubiquitin in a partially extended conformation where the residues 71 through 76 interact with some E2 residues proximal to the active site cysteine. The ubiquitin binding on E2 does not overlap the site where E3 enzymes will bind and is a transient binding allowing the transfer of the ubiquitin molecule to the substrate by the E3.
- **Third step/E3 ligases:** E3s are proposed as bridging factors that bring the E2 and the substrate together allowing for the covalent transfer of the ubiquitin molecule to the target protein and the subsequent formation of an

isopeptide link between the Gly76 of ubiquitin and a lysine residue of the protein (Passmore and Barford 2004; Pickart and Fushman 2004). There are two main families of E3 ligases characterized by the different domains that they contain:

  - E3s with HECT (Homologous to the E6-AP Carboxyl Terminus) domain which combine E2 and E3 activities (they bind themselves to ubiquitin before transferring it to the target).
  - E3s with RING (Really Interesting New Gene) domain consisting in a short motif rich in cysteine and histidine residues which coordinate two zinc ions allowing ubiquitination by positioning the protein E2 in the vicinity of the substrate and permitting the direct transfer of ubiquitin from E2 to the target.

- **Optional fourth step/E4 elongation ligases:** Once one ubiquitin moiety has been added to the target protein it exist the potential to be extended to form poly-ubiquitin chains by E2/E3 and/or thanks to the action of the E4 enzymes (Hoppe 2005).

The specificity of the E2/E3 interaction determines the final destinations (target protein) of the ubiquitin carried by a given E2 (Pickart and Fushman 2004; Varadan et al. 2004).

1.5 Types of Ubiquitination

Several types of protein modifications can be done using the ubiquitination reaction and the resulting functional alteration of the target protein will depend on the type of ubiquitination that it undergoes. This section describes the main characteristics and functions of the different types of ubiquitination: (1) the mono and multi-mono-ubiquitination and (2) the different types of poly-ubiquitination by ubiquitin chains (Fig. 4).

1.5.1 Mono and Multi-mono-ubiquitination

Monoubiquitination consists in the addition of one ubiquitin molecule to one lysine residue of a target protein. Multi-monoubiquitination consists in the addition of one ubiquitin molecule to multiple lysine residues of a target protein.

Monoubiquitination notably affects cellular processes such as histone regulation, endocytosis, DNA damage control and viral budding (Hicke 2001; Haglund et al. 2003a; Haglund et al. 2003b; Sigismund et al. 2004; Haglund and Stenmark 2006).

Multi-monoubiquitination of different membrane Tyrosine Receptors Kinase (TRKs) has been well studied (Huang et al. 2006). The detailed description of the specific function of mono and multi-mono-ubiquitination in membrane receptor endocytosis will be discussed further in the *chapter 4. Ubiquitin System in Endocytosis of Membrane RTKs.*

Besides the induction of endocytosis, mono- and multi ubiquitin linkage has a great potential to rearrange protein loops, induce higher order structures or stabilize the active conformation of proteins (Komander 2009; Komander and Rape 2012; Swatek and Komander 2016).

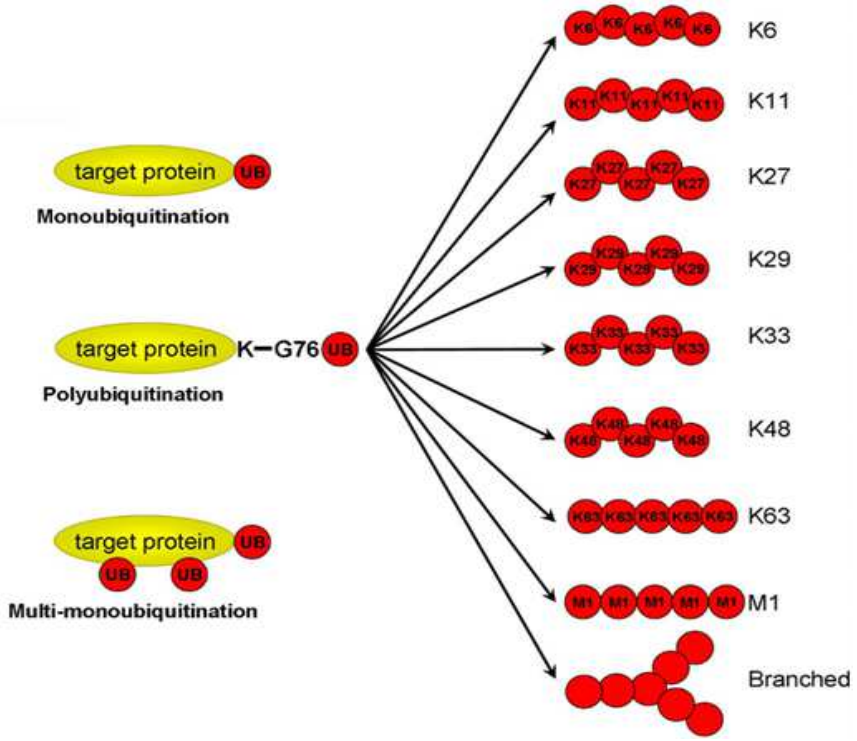


Figure 4: The different types of ubiquitination. Ubiquitination may occur once resulting in monoubiquitination, several times but on different Lys of the substrate, resulting in multi-monoubiquitination, and finally, several times but on the same Lys of the substrate, resulting in polyubiquitination. Depending on which Lys is involved in the chain formation, different types of polyubiquitin chains can be produced.

In the particular case of ubiquitin receptors (Ubiquitin binding domain (UBD) containing proteins), they can undergo monoubiquitination in a process called "coupled monoubiquitination": the presence in the same protein of at least one

UBD and one monoubiquitin modification site originates intramolecular interactions between the UBD and the ubiquitin leading to the auto-inhibition of the receptor (Hoeller et al. 2006).

EPS15-Nedd4 inter-regulation represents a well described example of regulation by the UBDs. Eps15 contains two UBDs: one of them binds to the ubiquitin of the ubiquitinated Nedd4 ligase, and this allows its subsequent ubiquitination by Nedd4. The newly added ubiquitin interacts with the second UBD stabilizing the opening of Eps15. Moreover, ubiquitinated Nedd4 exists as an inactive trimer (Attali et al. 2017), therefore, the interaction with EPS15 may somehow brake the trimer and induce Nedd4 activation. Current evidences suggest that such ubiquitin-mediated auto- and inter-inhibition play a major role in regulating many cell processes (Hicke et al. 2005; Sadowski et al. 2012).

On the contrary, ubiquitin linkage can triggers protein activation as illustrated in the two following examples. The first corresponds to the activation of the Ataxin3 enzyme: in order to become active, a large helical domain has to move to reveal the active site of the enzyme. Actually, ubiquitination within the N-terminal domain activates the enzyme. Hence, the authors speculate that the ubiquitination stabilizes this open conformation (Todi et al. 2009). Reviewed in Komander, 2009 #278).

The second example is the NEDDylation of the E3 enzyme Cullin which produces a large-scale intramolecular movement that activates the enzymatic reaction by stabilizing the transitory state (Saha and Deshaies 2008). The structure of NEDDylated Cul-5 was the first structural description of a protein conformation modified with ubiquitin-like molecules.

Recently, a new field of study of ubiquitination-dependent oligomerization is emerging but few examples have been reported so far (see *section 3.6 examples of regulation of oligomerization by ubiquitin*).

1.5.2 Poly-ubiquitination

The ubiquitin polypeptide has seven lysine residues exposed for further linkage of ubiquitin (Fig. 1) so several ubiquitin monomers can covalently bind to each other to form poly-ubiquitin chains on target proteins. The covalent link can be also done using the amino-terminal methionine of ubiquitin and will form linear ubiquitin chains (M1 chains) assembled by a special complex called Linear Ubiquitin Assembly Complex (LUBAC) (Komander and Rape 2012). Homotypic or heterotypic chains have been described and multiple homotypic chains on the same substrate have been

observed. Rreviewed in (Komander 2009; Swatek and Komander 2016). Moreover, on a structural point of view, the polyUb chains can be linear or not linear. Non-liner chains are (the numbers indicate the position of the lysine that it is used in the ubiquitin protein to elongate the chain):

- Poly-ubiquitin chains linked through lysine residue at position 48 (K48)
- Other non-linear chains: K6, K11, K27, K29 and K33
- Branched chains

Linear chains are:

- K63
- M1

Some lysine residues would be preferentially involved in poly-ubiquitination. A proteomic study in yeast showed that the poly-ubiquitin chains which are the more frequent are K48 (29% of all ubiquitin linkage in the cell) and K63 (17%) (Xu et al. 2009).

K48 chains where the first discovered and they are the main tag for proteasomal degradation. However, most of the other types of ubiquitin chains except K63 have also been reported to have proteasomal degradative function although less efficiently than K48 (probably indirectly by the interaction with transporters) in addition to some specific functions (Hershko and Ciechanover 1998; Xu et al. 2009; Dikic 2017). On its side, K63 ubiquitination is mainly implicated in cell signaling regulation and DNA repair (Ciechanover 2005; Chen and Sun 2009). Importantly, structural studies have shown that each of the two ubiquitin moieties in a K63 di-ubiquitin can bind UBD in the same mode as monoubiquitin does (Varadan et al. 2004).

Other ubiquitin chains found in this study were K6 (11%), K11 (28%), K27 (9%), K29 (3%) and K33 (3%). Also, mixed or branched chains have been reported as well as a new type of liner ubiquitin chain called M1 which were further shown to play important regulatory role particularly in the NFκB pathway activation (Iwai and Tokunaga 2009; Rittinger and Ikeda 2017). Moreover, heterologous chains formed by ubiquitin and SUMO proteins have been also described (Xu et al. 2009).

The specificity of the signal produced by each type of chain lies on the structure of the ubiquitin chain itself conditioning its recognition by the different UBDs (Hurley et al. 2006). For example, as mentioned above, K48 and K63 chains have completely different structure and though confer completely different type of protein modification (Fig. 5).

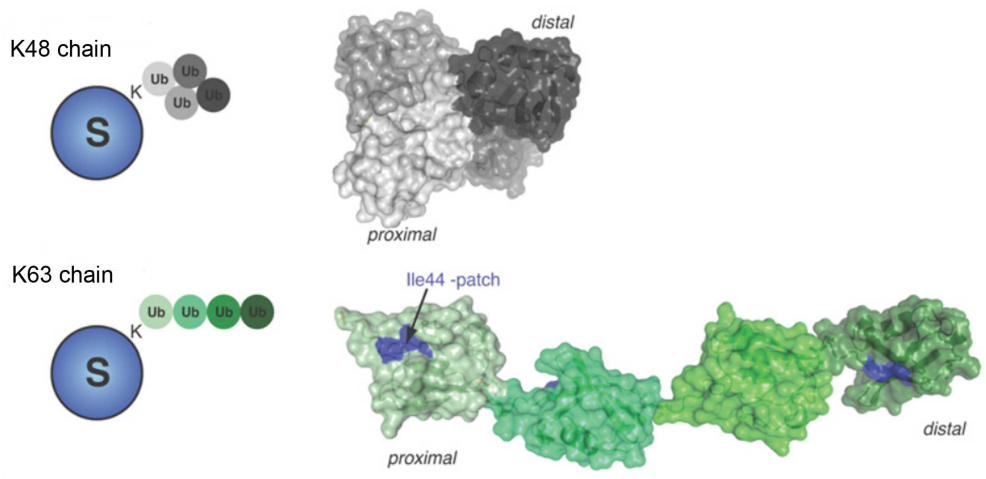


Figure 5: Structure models of K48 and K63 tetra-ubiquitin chains: Structure of **Lys48**-linked tetra-ubiquitin chain. Proximal (white) and distal (black) molecules are labelled. Proximal/distal describes the position relative to the substrate, see cartoon on the left. In Lys48-linked chains, all ubiquitin molecules interact with each other, and the Ile44 patches are not exposed. **Lys63**-linked ubiquitin chains display an open conformation, both in the crystal structure and in solution. The Ile44 patches (shown as blue surface on the molecules) are exposed, and can adopt different relative positions due to the flexibility in the ubiquitin chain. (Modified from Komander 2009).

1.6 Deubiquitination: the Role of DUBs

Protein ubiquitination is a reversible process thanks to the action of the deubiquitinases (DUBs) (Fig. 6). There are around 85 deubiquitinases encoded in the human genome (Komander et al. 2009; Leznicki and Kulathu 2017; Mevissen and Komander 2017) which ensure the renewal of ubiquitin pool on the one side and play regulatory role in plenty of processes like regulation of the proteasome function (Pickart and Cohen 2004), the chromatin structure (Yamashita et al. 2004), the endocytosis (Haglund et al. 2003b) and due to these wide range of action they are also implicated in pathological processes from hereditary or acquired cancer to neurodegeneration (Haas and Bright 1987; Amerik and Hochstrasser 2004; Nijman et al. 2005a; D'Arcy et al. 2015; Heideker and Wertz 2015; Perez-Rivas and Reincke 2016; Faucz et al. 2017; Min et al. 2017; Schmid et al. 2017).

1.6.1 DUBs Families

DUBs cleave ubiquitin or ULPs after the terminal carbonyl of the last Gly76. The existence of eight topologically different ubiquitin chains allows for differential

recognition by the DUBs. Much chain type-specific DUBs exist. They have been classically classified into six families based on their sequence similarity and mechanism of function (Fig. 7A): the ubiquitin specific proteases (USPs) is the largest family containing 55 members in the human genome, the ubiquitin carboxy-terminal hydrolases (UCHs), the ovarian-tumor related proteases (OTUs) and the Ataxin-3 proteins/Josephine domain proteases that are cysteine proteases containing the catalytic triad residues (Histidine, Aspartame and Cysteine) in their active site pocket. In the case of OTU their catalytic activity relies on the group thiol of the Cysteine which deprotonation is assisted by the Histidine and polarized by the Aspartate. The fifth family, the JAMM/MNP+ proteases, are metalloproteases which use a Zn2+ bound polarized water molecule for their proteolytic activity (Amerik and Hochstrasser 2004; Nijman et al. 2005b). OTU and JAMM/MNP metalloproteases present a very high specificity for K63 chains while USPs display low linkage selectivity (Ritorto et al. 2014). Recently, a new family of DUBs found in all eukaryotes has been discovered, the MINDY (motif interacting with ubiquitin-containing novel DUB family). MINDY-family are thiol proteases highly selective at cleaving K48-linked ubiquitin chains. The catalytic domain has no homology to any of the known DUBs. MINDY-1 prefers cleaving long polyUb chains and works by trimming chains from the distal end (Abdul Rehman et al. 2016).

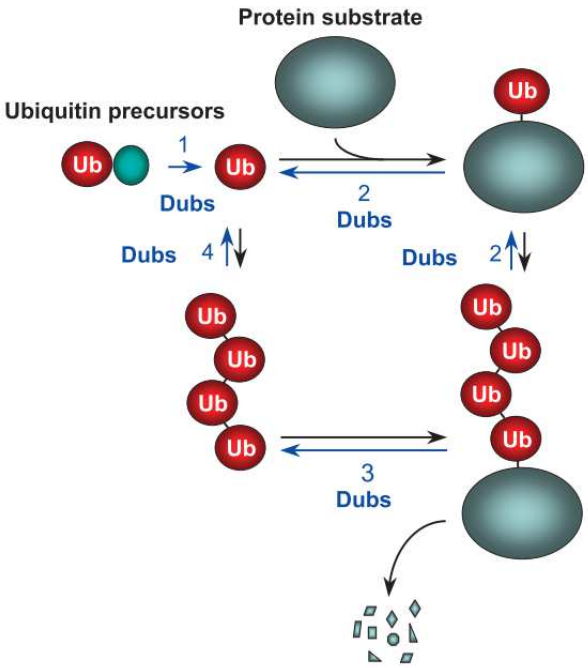


Figure 6: Functions of DUBs in the ubiquitin pathway. **(1)**Processing of ubiquitin precursors. **(2)** Editing or rescue of ubiquitin conjugates. **(3)** Recycling of ubiquitin or ubiquitin oligomers from ubiquitin–protein conjugates targeted for degradation. **(4)** Disassembly of unanchored ubiquitin oligomers. Modified from (Amerik and Hochstrasser 2004).

1.6.2 DUBs Specificity

In the case of DUBs, specificity can refer to the type of ubiquitin moieties itself (substrate specificity) (Fig. 7B) or to the target protein to which the ubiquitin is conjugated (target specificity). However, it is probable that in most of the cases a combinatorial mechanism of recognition of the target and the attached moiety determines overall DUB specificity within living cells. Rreviewed in (Mevissen and Komander 2017).

1.6.3 Regulation of DUBs Localization and Activity

The regulation of the catalytic activity and/or the specificity of DUBs is critical for maintaining cell integrity. In fact, overexpressing a DUB in a cell or in the organism is highly toxic due to unspecific deubiquitination of multiple substrates. DUBs are generally produced as active enzymes and therefore, the functional specificity of a given DUB is likely due to a very precise regulation of its activity through different layers of regulation. The subcellular localization of the DUB has a primary critical regulatory function which is integrated with control of expression, substrate activation, allosteric regulation and post-translational modifications (Reyes-Turcu et al. 2009; Coyne and Wing 2016).

Many DUBs contain different protein domains implicated directly or by association to other proteins in their specific subcellular localization (Coyne and Wing 2016). For example, in the case of UBPY, the MIT N-terminal domain has been implicated in its recruitment to the endosomal membranes, upon stimulation of human cell culture with EGF, by interaction with members of the endocytic machinery (Row et al. 2006). Another example is the differential localization of the different isoforms of USP36 in *Drosophila*. USP36 isoforms have either nucleolar or cytoplasmic localization depending on the presence of specific hydrophobic domain allowing the export of the protein from the nucleus. DUB specific localization will limit the available substrate and will determine its function either in the regulation of transcription (Thevenon and Taillebourg, unpublished observations) or in the immune deficiency (IMD) signaling pathway in *Drosophila* (Thevenon et al. 2009). In human cells, USP36 is expressed in the nucleolus where it regulates the nucleolar structure and ribosomal function (Endo et al. 2009).

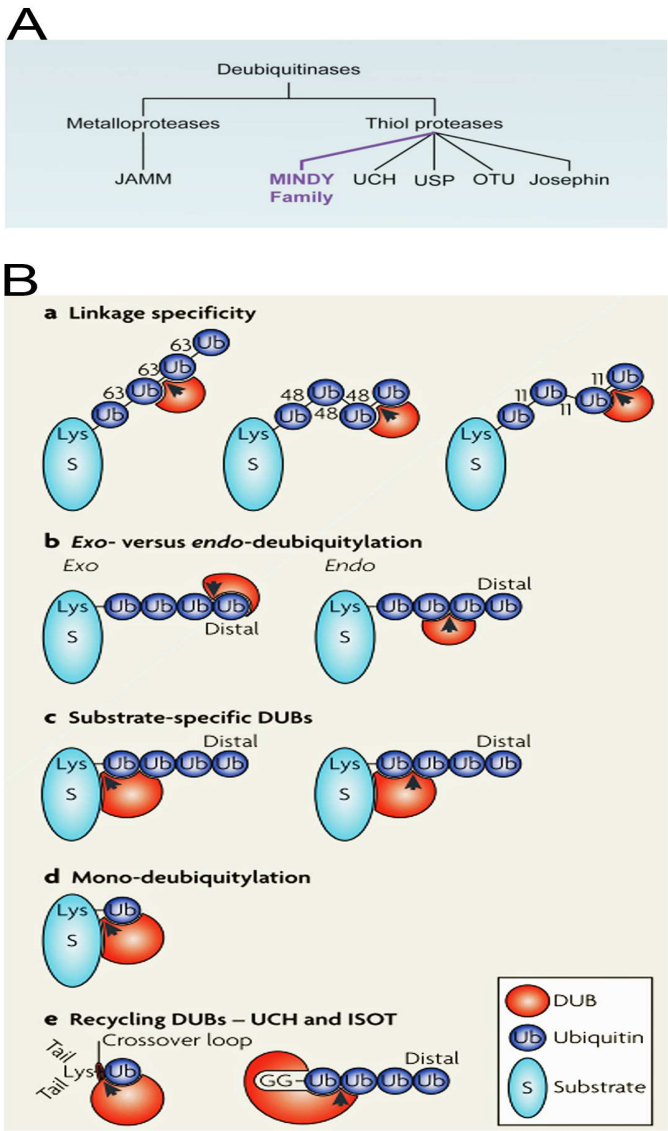


Figure 7: DUBs families and specificity towards Ubiquitin substrates. **A:** DUB families. **B: (a)** Chain type-specific DUBs **(b)** In ubiquitin chains, cleavage can occur from the ends (exo) or within a chain (endo). **(c)** DUBs may be targeted to their substrates directly **(c right)** Promiscuous DUBs might remove ubiquitin completely from substrates. Alternatively, they might leave the substrate monoubiquitinated **(d)** DUBs might specifically recognize their cognate protein substrate to remove monoubiquitin **(e left)** UCHs can effectively remove small disordered sequences from the C terminus of ubiquitin **(e right)** DUBs are also responsible for the recycling of free ubiquitin from unattached ubiquitin chains. **Cleavage (shown by the arrows).** Modified from (Nijman et al. 2005b).

Other regulatory mechanisms controlling DUBs specificity can be (non-exhaustive list):

(1) Proteasomal degradation or transcriptional regulation controlling DUBs abundance in the cell. Transcriptional regulation has been notably reported for USP1 or CYLD, beside others. In the case of CYLD, it serves as a retro-negative control of NF-kB pathway activation (Jono et al. 2004; Nijman et al. 2005a; Reiley et al. 2005).

(2) Some UCHs and USPs (like USP8/UBPY, USP7 or USP14) are only active when they are bound to ubiquitin (Hu et al. 2002; Hu et al. 2005) or highly activated by association to the proteasome like USP14 (Hu et al. 2005; Lee et al. 2016b). In the case of USP7, the free active site is in an open non-functional conformation due to

non-functional alignment of the catalytic triad. Upon interaction with the ubiquitin substrate, a conformational change takes place and the enzyme becomes catalytically active (Hu et al. 2002; Renatus et al. 2006).

(3) In many examples, association into higher-order structures are necessary for the activation or inhibition of the catalytic activity of the DUBs. Most of the times UBD present in the DUBs or in their partners play an important role in the formation of the complexes. Being part of the complex can regulate the deubiquitinase activity by changing its localization and access to substrates like in the case of AMSH recruitment to the endosomes (McCullough et al. 2006) or USP14 recruitment to the proteasome (Borodovsky et al. 2001). Alternatively, in the case of USP10, its association to Ras-GTPase activating protein (G3BP) inhibits the ability of USP10 to disassemble ubiquitin chains (Soncini et al. 2001; Komander et al. 2009).

(4) In some cases, cleavage of the DUB by proteolysis is necessary for its activation (for example of USP7 and A20) (Vugmeyster et al. 2002; Coornaert et al. 2008).

(5) Post-translational modification as phosphorylation or ubiquitination can also regulate the catalytic activity of the DUBs like in the case of CYLD (Reiley et al. 2005) and ataxin-3 (Todi et al. 2009).

(6) The oxidative stress has been also reported to regulate the activity of some DUBs (Enesa et al. 2008).

It is important to highlight the presence of ULDs in many DUBs, especially into the USP family. These domains can regulate DUB activity by binding to the UBD of partner proteins (like in the case of USP14 interaction with the proteasome which strongly enhances its activity). But they can also bind to DUB' UBD and negatively regulate the enzyme by competition with the substrate for the catalytic site (like in the case of USP4), or positively regulate the DUB' activity by promoting conformational changes that allows the binding of ubiquitin to the catalytic site (like for USP7) (Faesen et al. 2011; Faesen et al. 2012).

For a recent review in DUBs regulation see (Mevissen and Komander 2017) and (Leznicki and Kulathu 2017).

1.7 Interpretation of the Modification: Ubiquitin-Binding Domains (UBDs) or Ubiquitin Interacting Motifs (UIMs)

Ubiquitin modifications provide a larger and chemically varied surface areas with the enclosed potency of multifaceted cellular interpretations. Precise

interpretation of the signals mediated via ubiquitin or ULP modifiers depends on the correct employment of a large family of ubiquitin or UBL binding domains (UBDs). This feature has promoted the coevolution of ULPs, on the one hand, and a multitude of UBDs, on the other hand. There are more than 16 so far characterized UBDs. They are rather small (20-150 amino acids) and diverge in both structure and patterns of ubiquitin recognition (Hurley et al. 2006; Grabbe and Dikic 2009; Low et al. 2013). Beside, more than 150 proteins contain one or more UBD domains (Hicke et al. 2005; Dikic et al. 2009).

In most cases the binding between the UBDs and ubiquitin is of low or moderate affinity characterizing ubiquitin-mediated protein interactions as flexible and highly dynamic.

So far *in vitro* experiments do not show that specific UBDs have any preference toward any type of ubiquitin linkage. Instead, specificity appears to be conveyed by surrounding domains within the same protein or another protein within a larger protein complex, namely, by location (Grabbe and Dikic 2009; Low et al. 2013; Coyne and Wing 2016).

As described above, UBD-containing proteins have a predilection to become ubiquitinated themselves, an event mediated via a process known as "coupled monoubiquitination". This unique type of ubiquitination is dependent on the presence of a functional UBD and in many cases, it is independent from any E3 ligase (Hoeller et al. 2007).

A subgroup of proteins combine a ULD with an UBD within the same polypeptide chain, endowing the resulting protein with unique properties, such as intramolecular interaction and autoregulation (Grabbe and Dikic 2009).

1.8 Evolution and Conservation of the Ubiquitin System

The origin of the ubiquitin system dates back to the archaea kingdom. There is no functional analogue in the prokaryote kingdom although there are proteins with similar fold and similar function, notably among pathogenic bacterial effectors that may have co-evolved with eukaryotic host (Pickart and Eddins 2004; Ashida and Sasakawa 2017; Delley et al. 2017; Lin and Machner 2017).The bacterial species ThiS and MoaD have C-terminal G-G motifs which undergo reactions similar to ubiquitin activation, however, these proteins transfer sulfur rather than conjugate to other proteins (Kessler 2006). Only a prokaryotic ubiquitin-like protein, Pup, was found in *Mycobacterium tuberculosis* to target proteins for proteolysis by the proteasome

(Darwin 2009). In the archaea, the ubiquitin system was identified in the single sequenced genome from the new phylum *Calycomorphum subterraneum*. In its genome, on can find genes coding for one ubiquitin, one ubiquitin ligase and a key deubiquitinase that clearly cluster with the eukaryotic homologous genes (Nunoura et al. 2011). All the ubiquitin-related genes, including SUMO and Ifm1 were already present in the eukaryotic common ancestor (Grau-Bove et al. 2015).

During the process of eukaryogenesis that led from the First Eukaryotic Common Ancestor (FECA) to the common ancestor of all the eukaryotes known as Last Eukaryotic Common Ancestor (LECA), a rapid expansion of the ubiquitin-toolkit genes happened which has been hypothesized to be a key mechanism for the simbiogenic origin of eukaryotes and the control of exogenous membranes (Grau-Bove et al. 2015; Koonin 2015b; Koonin 2015a). Later on, the LECA increased its multi-domain protein families at the same time that it increased the connected ubiquitin domain architecture network. Both events accompanied the appearance of the endo-membranes system and the intracellular vesicle trafficking. This contemporaneity could lead to speculate the interdependence of both systems from the origin of its evolution (Grau-Bove et al. 2015).

During multicellularity evolution, gene expansion affected mostly the E3 ligases and the deubiquitinases increasing substrate specificity and leading to functional diversification to reach the maximal complexity in the metazoan (animals and plants) (Semple 2003; de Mendoza et al. 2013). Ubiquitin, Nedd8 and SUMO are distributed among all the eukaryotes but other UBL like Ufm1 are mammalian newly inventions (Pickart and Eddins 2004).

2. ENDOCYTOSYS

2.1 Introduction

Eukaryotic cells are able to intake macromolecules and particles from the surrounding medium by endocytosis. Endocytosis also regulates the levels of many essential surface proteins and transporters including plasma membrane receptors like G-protein coupled receptors and receptor tyrosine kinases (RTKs). In addition, endocytosis regulates cell-cell and cell-matrix interactions through uptake of integrins and adhesion molecules (Goh and Sorkin 2013; Elkin et al. 2016).

In endocytosis, the material to be internalized is surrounded by an area of plasma membrane which then buds off inside the cell to form a vesicle containing the ingested material. The term "endocytosis" was coined by Christian deDuve in 1963 to define both the ingestion of large particles (such as bacteria), -which is now rather termed phagocytosis-, or the uptake of fluids or macromolecules in small vesicles.

The endocytic pathways include clathrin-mediated, caveolae/lipid raft-mediated, clathrin-, and caveolae-independent endocytosis, fluid-phase endocytosis, and phagocytosis (Fig. 8). They differ in the nature of internalized cargoes, the size of vesicles, the associated molecular machinery, and the type of regulation. For review, see (Conner and Schmid 2003) and (Merrifield and Kaksonen 2014).

The two main types of endocytosis are:

- phagocytosis
- pinocytosis

During **phagocytosis**, cells engulf large particles such as bacteria, cell debris, or even intact cells. Binding of the particle to receptors on the surface of the phagocytic cell triggers the extension of pseudopodia (an actin-based movement of the cell surface). The pseudopodia eventually surround the particle and their membranes fuse to form a large intracellular vesicle (>0.25 µm in diameter) called a phagosome. The phagosomes then fuse with lysosomes, producing phagolysosomes in which the ingested material is digested by the action of lysosomal acid hydrolases. During maturation of the phagolysosome, some of the internalized membrane proteins are recycled back to the plasma membrane (see receptor-mediated endocytosis). Amoebas use phagocytosis to capture food particles such as bacteria

or other protozoans. In multicellular animals, the major roles of phagocytosis are to provide a defense against invading microorganisms and to eliminate aged or damaged cells from the body. Macrophages and neutrophils are the main cells in charge of phagocytic immune function in mammals.

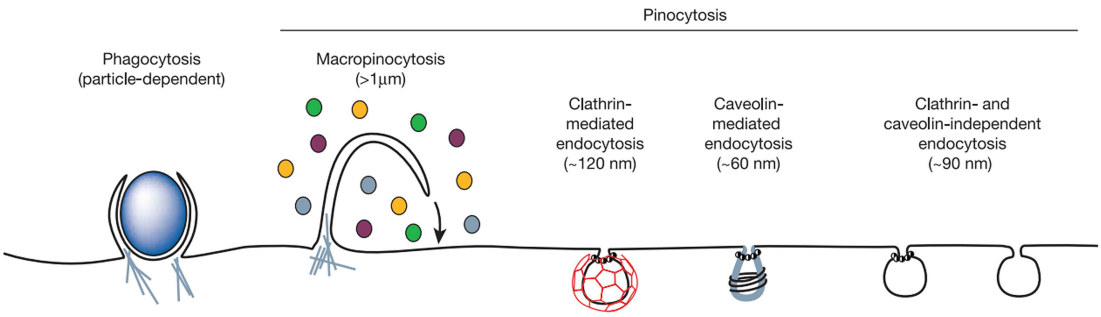


Figure 8: Types of endocytosis in mammalian cells: The endocytic pathways. Modified from (Conner and Schmid 2003).

Pinocytosis is the uptake of fluids or macromolecules in small vesicles. It is actin-independent and the two main pathways are either clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE or caveolae/lipid raft-mediated). Selective inhibition of these two pathways led to the discovery of cholesterol-sensitive clathrin and caveolae-independent pathways, and more recently, the large capacity pathway involving clathrin independent carriers (CLIC) and glycophosphatidylinositol anchored protein-enriched endosomal compartments (GEEC), termed the CLIC/GEEC pathway. The Clathrin-Calveolin independent endocytosis can be dynamin dependent or independent. Reviewed on (Elkin et al. 2016).

2.2 Receptor-Mediated Endocytosis

A very well characterized form of pinocytosis is the receptor-mediated endocytosis of specific cell surface receptors such as receptors for chemokines, cytokines or growth factors. Following activation, these receptors are concentrated in specialized regions of the plasma membrane and then internalized into the cell. The endocytosis of plasma membrane receptors can be either CME or CIE and the

difference in the internalization mode will set up the destiny of the endocytosed material. I will focus the rest of the chapter in this kind of endocytosis.

2.2.1 Internalization

2.2.1.1 Clathrin-Mediated Endocytosis (CME)

CME consists in the concentration of transmembrane receptors and their bound ligands into 'coated pits' on the plasma membrane. Coated pits are formed by the assembly of cytosolic clathrin and they are known as clathrin-coated pits (CCPs). CCP assembly is initiated by the hetero-tetrameric adaptor protein 2 (AP2) complexes that are recruited to the plasma membrane and rapidly recruit clathrin. The AP2 complexes are formed by four subunits of AP2 complexes and link the clathrin coat to the lipidic membranes. AP2 is also a main cargoes-recognition and concentration factor and is responsible for recruiting other adaptor proteins. Concomitant clathrin assembly of the receptors and recruitment of the adaptor proteins will produce invagination of the CCPs (Kirchhausen et al. 2014).

The detachment of the CCPs from the plasma membrane (PM) to release mature clathrin-coated vesicles (CCVs) depends on the large GTPase dynamin. Dynamin assembles into collar-like structures around the necks of deeply invaginated pits and undergoes GTP hydrolysis to drive membrane fission. CCVs are encapsulated by a polygonal clathrin coat and carry concentrated receptor–ligand complexes. The clathrin coat is disassembled by the ATPase, heat shock cognate 70 (HSC70) and its cofactor auxilin; this allows the uncoated vesicle to travel and fuse with its target endosome (Fig. 9) (Conner and Schmid 2003; Elkin et al. 2016).

There are "hot-spots" for the formation of the CCVs in actin-rich regions. Spatial and temporal regulation of CME depend on the nature of the receptor, the ligand and their interaction but also on a wide variety of interactions of the receptor-ligand complex, the clathrin and the AP2 with adaptors proteins (Eps15, amphiphysin, intersectin beside others) and by phosphorylation cascades (Wilde and Brodsky 1996; Conner and Schmid 2003; Cocucci et al. 2012; Elkin et al. 2016).

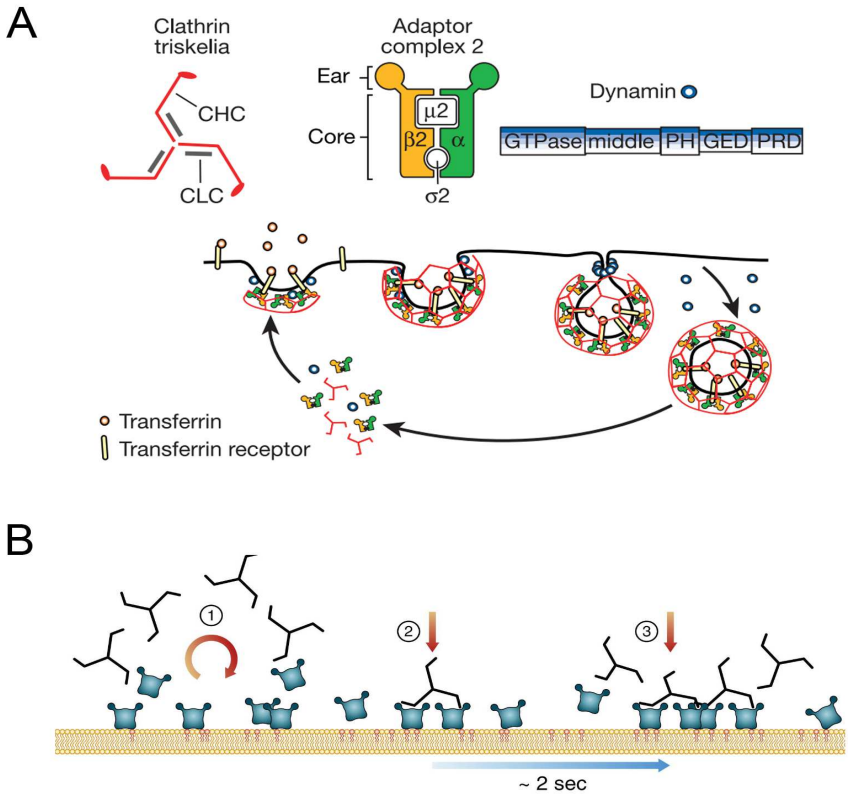


Figure 9: CME process. A: Schematic representation of three core components of the machinery driving CME of the transferrin receptor: Clathrin, AP2 and Dynamin. **B:** Recruitment of Clathrin and AP2 during the first five seconds of CCPs formation. Modified from (Conner and Schmid 2003) and (Cocucci et al. 2012).

2.2.1.2 Clathrin-Independent Endocytosis (CIE)

The term CIE encompasses several pathways of which the caveolae-mediated endocytosis (CavME) is the best characterized and studied. CavME is highly regulated and triggered by ligand binding to cargo receptors concentrated in caveolae (Parton and Simons 2007). The main structural proteins of caveolae is caveolin-1 (a small integral membrane protein that is inserted into the inner leaflet of the membrane bilayer), in co-assembly with Cavins (cytosolic coat proteins). The cytosolic N-terminal region of caveolin-1 binds to cholesterol and functions as a scaffolding domain that binds to important signaling molecules (Parton and del Pozo 2013; Nassar and Parat 2015). Caveolae are static structures that bud from the plasma membrane and are regulated by kinases and phosphatases. Like for CME, dynamin is necessary to liberate caveolae vesicles from the plasma membrane

(Henley et al. 1998; Kiss 2012). But differently from clathrin, uncoating the vesicle is not necessary for its fusion with its target endosome. CavME is important in transcytosis, mechano sensing, lipid regulation, control of cell surface tension and act as signaling platforms (Parton and Simons 2007; Parton and del Pozo 2013).

Other CIE pathways are:

(1) an endophilin-, dynamin- and RhoA-dependent pathway that mediate uptake of many cytokine receptors and their constituents (Boucrot et al. 2015).

(2) A clathrin- and dynamin-independent pathway that involves small GTPases termed the CLIC/GEEC pathway (Mayor et al. 2014).

(3) A pathway depending on the small GTPase, Arf6, that mediates uptake and recycling of the Major Histocompatibility Antigen I (Donaldson and Jackson 2011; Mayor et al. 2014).

(4) a pathway dependent on curvature-generating, membrane-anchored proteins, called flotillins (Glebov et al. 2006; Frick et al. 2007) and a super-fast endophilin-mediated endocytosis (FEME) (Boucrot et al. 2015).

2.2.2 Intracellular Trafficking

2.2.2.1 General features

Once internalized into the cell, receptors and their bound ligands merge into the common endosomal network. The endosomal network collect internalized cargoes, sort, and disseminate them to their final destinations (Huotari and Helenius 2011; Preston et al. 2014). Primary endocytic vesicles form the early/sorting endosomes. Early endosomes maturate to late endosomes and ultimately the fates of the internalized receptors are decided. Actually, the so-called cargoes can be recycled back to the plasma membrane, sent to the trans-Golgi network (TGN) *via* retrograde traffic, notably in polarized cells, sent across the cell through a process called transcytosis or sorted to the lysosome for degradation (Fig. 10) (Raiborg and Stenmark 2009; Preston et al. 2014).

The regulation of membrane trafficking and cargoes sorting is achieved through the tight spatial and temporal control of endosomal identity. These identities follow fluidics laws and can be altered as molecules and cargoes are dynamically exchanged. Changes in endosomal identity are defined as endosomal maturation.

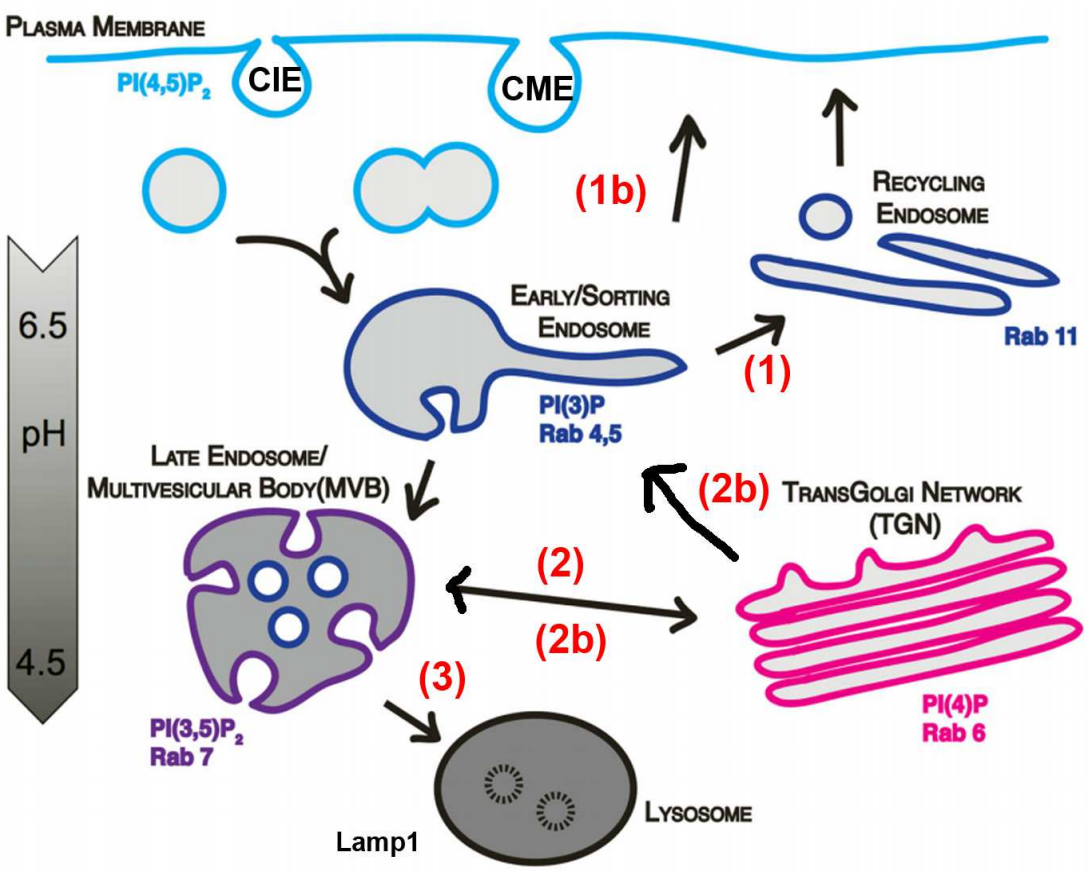


Figure 10: Endosomal trafficking. CME or CIE internalized vesicles undergo fusion to form early/sorting endosomes, which tabulate to facilitate cargoes sorting: (1) recycled back to the plasma membrane through the recycling endosome or (1b) directly through the fast recycling pathway, (2) sent to the trans-Golgi network (TGN) via retrograde trafficking mechanisms, (2b) and from the TGN can be send back to the MVBs or to the sorting endosomes, and (3) trafficked through the late endosome/multivesicular body (MVB) to the lysosome for degradation. As endosomes mature they increase in acidification (depicted in greyscale). Additionally, each endosomal compartment is defined by signature of phosphatidylinositol phospholipids (PIPs) and Rab family GTPases. Modified from (Elkin et al. 2016).

It is probable that none of the trafficking mechanisms is 100% efficient, so molecules will always be found, to some extent, in alternative pathways. Moreover, all of the compartments are dynamic, and most of the molecules are never permanently resident in any compartment, which makes very difficult to use good compartment markers and therefore the study of the intracellular trafficking.

2.2.2.2 Endosome Identity, Function, and Trafficking

During maturation the lumen of the endosomes becomes increasingly acid. This feature plays a major role in the control of the directional flow of membrane trafficking by alteration of key phosphatidylinositol lipids (by lipid kinases and phosphatases), and by differential recruitment and activation of Rab-family GTPases (Mellman et al. 1986).

Specific PIPs are enriched at distinct compartments (Bissig and Gruenberg 2013). This change in lipid distribution is important for the differential recruitment of proteins involved in trafficking to specific membrane compartments through PIP-specific lipid binding domains. AP2 and dynamin, selectively bind to PI(4,5)P2, which is enriched at the plasma membrane. FYVE domains and PhoX domains preferentially bind PI(3)P (Balla 2005), which is mostly restricted to early endosomes and intraluminal vesicles (ILVs) (Fig. 10) (Gillooly et al. 2000; Klumperman and Raposo 2014).

There is a close relationship between PIP modifying enzymes and Rab GTPases. For example, PIPs can recruit regulators of Rab proteins that activate or inactivate Rabs on target membranes. In addition, PIP kinases and phosphatases are often Rab effectors (Odorizzi et al. 2000) and many of these Rab effectors have PI(3)P binding motifs, including early endosomal antigen 1 (EEA1 through its FYVE domain). Thus, PIPs and Rabs can coordinate membrane identity through both positive and negative feedback mechanisms (Zerial and McBride 2001; Shin et al. 2005).

Rab Proteins:

Rab GTPases are small molecular weight monomeric guanine nucleotide binding proteins. Rab GTPases function as "molecular switches" and cycle between GDP-bound inactive and GTP-bound active states. They require other proteins to modulate their GTP-GDP cycle, i.e., the guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) which catalyze the GTP exchange and hydrolysis reactions, respectively. Endomembrane identity, control of vesicle formation and targeting, and fusion of endosomes are processes all dependent on the association of an active Rab GTPase and its effector protein(s) (Zerial and McBride 2001; Stenmark 2009; Pfeffer 2013; Wandinger-Ness and Zerial 2014).

The Rab protein complexes are spatially and temporally regulated through the GTP-GDP cycle. The Rab cascade concept is the prevalent model: an upstream Rab recruits a GEF that activates and recruits a downstream Rab which in turn recruits a GAP for the upstream Rab and in this way, converts the membrane's

identity (Novick and Zerial 1997; Zerial and McBride 2001). This regulation allows directionality of endocytic transport, endomembrane identity and the fidelity of cargoes transport (Gurkan et al. 2005). Reviewed in (Pfeffer 2013; Pfeffer 2017).

2.2.3 Mechanisms of Endosomal Sorting

The receptor-ligand complex contained in the newly formed endosomes enters the endosomal network by fusion with pre-existing sorting endosomes. Sorting endosomes are peripherally located and tubular-vesicular structures that have a luminal pH of ~6.0 (Johnson et al. 1993).

The mechanism of **fusion** with pre-existing sorting endosomes is regulated by Rab5, early endosome antigen 1 (EEA1) and SNAREs and depends on the characteristics of the newly formed vesicle which itself depends on the type of the internalization pathway (Clague 1999; Mills et al. 1999).

From the early sorting endosome there are three known destinations: the plasma membrane, the late endosomes/lysosomes and the TGN. There are two main routes back to the cell surface from sorting endosomes. Some cargoes are sent directly back to the plasma membrane (Fig. 10-1b) and others are delivered to a long-lived endocytic recycling compartment (Fig. 10-1).

Different passive (accessory protein independent) or active (accessory protein dependent) mechanisms are implicated in sorting:

2.2.3.1 Passive Sorting

The initial early endosomal sorting depends on **endosomal acidification** and ligand dissociation. Internalized receptor-ligand complexes have different pH sensitivities for dissociation. Receptors that recycle to the plasma membrane typically release their ligands in the early endosome, where the pH is maintained at ~6.5 (Fuchs et al. 1989) allowing for a fast recycling. If the destiny of the cargoes is the trans-Golgi network, the release of their ligands happens in the late endosomal compartment at pH range of ~5.5. To highlight this case: the EGFR-EGF (see below) and other signaling receptors very often remain ligand bound and active even at low (~4.5) pH allowing for their continual signaling on endosomal platforms until they are sorted into ILVs and eventually sent for degradation to the lysosome (Mellman 1996).

The main passive mechanism of sorting at the endosomes is **geometry-based sorting**: as early endosomes mature, they can exhibit tubulation, this structure facilitates cargoes sorting and recycling. Indeed, the narrow tubules that extrude from early endosomes have a maximum surface area-to-volume ratio which allows for accumulation of cargoes destined for recycling. Consequently, in the absence of specific targeting information, a membrane protein will be transported from the sorting endosome together with the bulk of the membrane. On the contrary, delivery of membrane proteins to late endosomes requires specific targeting information and is an ubiquitin dependent process (Maxfield and McGraw 2004).

Several Endosomal Sorting Complex Required for Transport (ESCRT) proteins have been described to form tubular structures both from synthetic liposomes and in human cells (McCullough et al. 2015), and therefore, the idea that the ESCRT proteins are implicated in the formation of recycling endosomes have been focused of attention during the last two years (See *chapter 3. ESCRT proteins*)(Schoneberg et al. 2017).

2.2.3.2 Active Sorting and Biogenesis of MVB

Interactions of cargoes with the different members of the sorting machinery is necessary for the sorting from early to late endosomes, and also for the sorting and/or recycling at the level of the late endosomes (Hsu et al. 2012).

Sorting nexin family proteins (SNXs) interact with endosomal membranes, induce its tubulation and control cargoes sorting as part of the evolutionarily conserved retromer complex which is required for retrograde transport (Hsu et al. 2012; van Weering et al. 2012; Burd and Cullen 2014). The retromer complex is recruited to endosomes through SNX-mediated PI(3)P binding and/or association with Rab7-positive late endosomes. They function to rescue cargo receptors from the degradative lysosomal pathway and divert them for recycling to the plasma membrane (Burd and Cullen 2014).

Other mechanisms of active cargoes sorting within the endosomal network which is specific of signaling receptors is the sorting of ubiquitinated cargoes into intraluminal vesicles (ILVs) for lysosomal degradation. When the receptor is ubiquitinated and targeted for degradation, the early endosomes mature and accumulate ILVs in their luminal portions by the inward budding of the limiting membrane becoming multivesicular bodies (MVBs) (Stahl and Barbieri 2002; Hurley 2010).

The formation of the ILVs requires three different steps: 1-Cargoes recognition and sorting. 2-Membrane budding. 3-vesicle fission from a donor membrane (endosomal membrane) (Gruenberg and Stenmark 2004). The formation of the ILVs have reversed topology than the endosomal or exosomal vesicles. ILVs bud away from the cytoplasm into the lumen of the MVB. Thereby, cargoes proteins are sequestered inside the MVB and removed from the cytoplasm. Sorting into the MVBs is mediated by the multi-subunit ESCRT machinery. Reviewed in (Hurley 2010; Babst 2011; Henne et al. 2013).

The specific role of ESCRT machinery in ILVs formation will be further described through the example of EGFR sorting in the *chapter 3. ESCRT Proteins and section 4.4.3.2 Sorting of the EGFR*. A general description follows:

In mammalian cells, ILVs are formed/generated at the edges of the clathrin-coated regions of the endosomal membrane, suggesting that clathrin disassembly must occur before ESCRT machinery assembly (Sachse et al. 2002; Murk et al. 2003). Furthermore, unlike in yeast, there are evidences for several additional ESCRT-independent machineries that can drive ILVs formation like the lipids LBPA (2,2'-dioleoyl lysobiphosphatidic acid) and ceramide (Matsuo et al. 2004; Trajkovic et al. 2008). Rreviewed in (Bissig and Gruenberg 2013). Nevertheless, most of the factors involved in ILVs formation have been initially identify in yeast as the vacuolar protein sorting (VPS) genes (Raymond et al. 1992).

MVBs were initially described by Sotelo and Porter in mammals (Sotelo and Porter 1959). MVBs are globular vacuoles of 250–1000 nm in diameter that contain different number of ILVs and display membrane tubules representing various recycling pathways. MVBs contain some patches of clathrin coat, indicating that protein sorting continue at this stage (Sachse et al. 2002; Klumperman and Raposo 2014). The presence of increasing number of ILVs into the MVBs reflects the temporal progression along the endo-lysosomal pathway from early to late endosomes/MVBs (Fig. 11). Reviewed in (Hanson and Cashikar 2012).

Multiple machineries driving ILVs formation result in multiple types of MVBs. Mainly two populations of MVBs exist which are morphologically identical but molecularly distinct: one cholesterol rich and another with only low amounts of cholesterol. Interestingly, the cholesterol-enriched MVBs fuse more frequently with the plasma membrane (Möbius et al. 2002).

The best studied example comes from the case of dendritic cells (Attali et al.). Rreviewed in (ten Broeke et al. 2013). In DC, the sorting of MHCII into ILVs depends on the cell activation status. In inactive DCs, MHCII sorting is ubiquitin-

dependent and results in lysosomal degradation (van Niel et al. 2006). In activated DCs, MHCII sorting is ubiquitin-independent and results in sorting to ILVs of smaller-sized generating MVBs with high ability to fuse with the PM and secrete their ILVs as **exosomes** (Buschow et al. 2009). These data indicate the presence of at least two populations of MVBs: one triggering lysosomal targeting and the other triggering **exosome secretion.**

2.2.4 Lysosomal Degradation

Cargoes are delivered from MVBs to lysosomes by heterotypic MVBs-lysosome membrane fusion; as shown in yeast, this step is preceded by the tethering of the two compartments by homotypic fusion and vacuole protein sorting (HOPS) complex and the trans-SNARE complex containing Syntaxin7 in coordination with ESCRTs (Luzio et al. 2009).

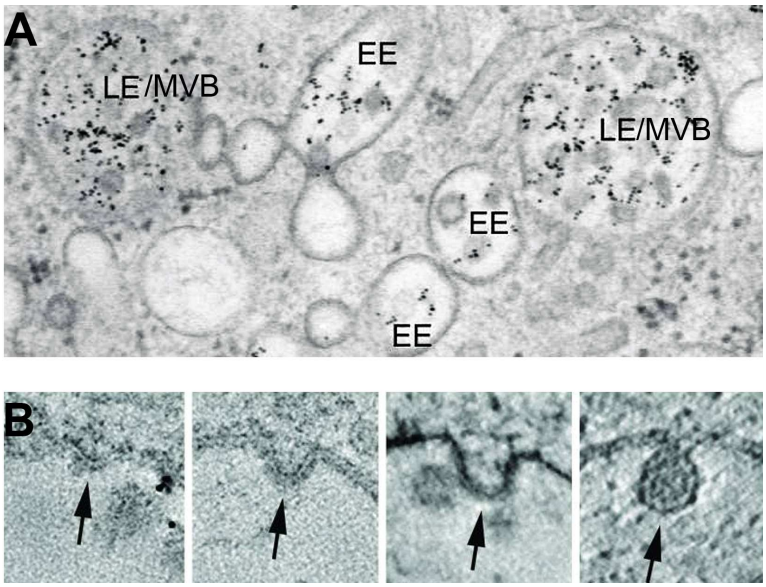


Figure 11: EM architecture of EEs, MVBs and ILVs formation. A: HepG2 cells pre-incubated for 3 h with BSA conjugated to 5-nm gold (BSA5). Both early endosomes (Baietti et al.) and late endosomes (LEs) are indicated. EEs have some ILVs fully formed or in formation. MVBs contain multiple ILVs**. B:** ILVs formation**.** Modified from (Klumperman and Raposo 2014).

The delivery of the cargoes from the MVBs to the lysosomes then happens by direct fusion or a kissing-and-run process (Fig. 12). In the lysosomes, the ILVs

are exposed to and degraded by lysosomal hydrolyses (lipases and proteases), and then a novel separation into the original parent compartments would occur. This is referred to as the "kiss and run" model (Wiley 2003; Luzio et al. 2009).

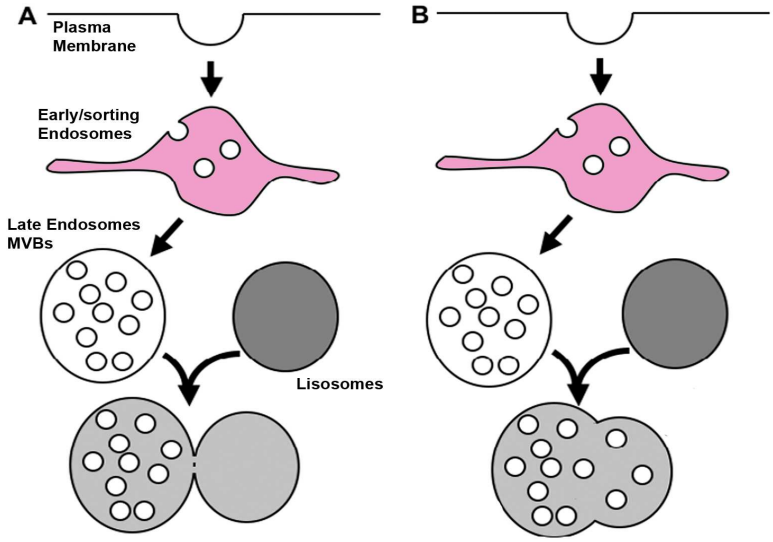


Figure 12: Models of MVBs-Lysosomes fusion. **A:** Kiss and run**. B:** Direct fusion. Lysosomes are regenerated from the subsequent hybrid organelles by a maturation process Modified from (Luzio et al. 2009).

3. ESCRT PROTEINS

3.1 Introduction

The Endosomal Complex Required for Transport (ESCRT) was first discovered in yeast as a set of 13 Vps class E genes required for proteins sorting in the MVBs (Raymond et al. 1992; Cereghino et al. 1995). In mammals, the first description of receptors sorting process mediated by ESCRTs was the work of (Katzmann et al. 2001; Babst et al. 2002a; Babst et al. 2002b; Katzmann et al. 2002). The functional conservation between Vps class E proteins in yeast and the mammalian ESCRT machinery has been demonstrated by many groups; a correspondence between the different ESCRT subunits and associated proteins in yeast and mammals is shown below (Table 1) (Babst et al. 2000; Fujita et al. 2003; Peck et al. 2004; Hurley 2010).

Table 1: ESCRT subunits and associated proteins

Complex	Yeast Protein	Mammalian Name	Selected Interactors*	Cellular Functions**
ESCRT-0 *Clustering of ubiquitinated cargoes*	Vps27	HRS	Clathrin, Eps15b,PIP3, Ubiquitin, TSG101	MVB, ATG
	Hse1	STAM1,2	Ubiquitin, AMSH, UBPY	MVB, ATG
ESCRT-I *Membrane budding (with ESCRT-II)*	Vps23	TSG101	Ubiquitin, HRA, Viral Gag proteins, CEP55	MVB, ATG, VB, CTK
	Vps28	VPSs28	Fps22, Vps36	MVB, ATG, VB, CTK
	Vps37	VPS37A,B,C,D	Lipidic membranes, IST1(only mammals)	MVB, ATG, VB, CTK
	Mvb12	MVB12A,B	Ubiquitin, all the ESCRT-I subunits	MVB, ATG
ESCRT-II *Membrane budding (with ESCRT-I)*	Vps22	EAP30	Lipidic membranes, Vps28	MVB, ATG
	Vps25	EAP20	CHMP6	MVB, ATG
	Vps36	EAP45	Ubiquitin, lipidic membranes, Vps28	MVB, ATG
ESCRT-III *Membrane scission*	Vps20	CHMP6	CHMP6, CHMP4, VPS4, Vps20, Vps28	MVB, ATG
	Vps24	CHMP3	CHMP3, CHMP4, CHMP2A, AMSH, UBPY, VPS4, Vta1	MVB, ATG, VB, CTK

	Vps2	CHMP2A,B	CHMP2, CHMP3, VPS4, Vta1, AMSH, UBPY,	MVB, ATG, VB, CTK
	Vps32/Snf7	CHMP4A,B,C	CHMP4, CHMP6, CHMP3, CHMP7, Bro1 domain containing proteins (Alix), UBPY (in yeast), VPS4	MVB, ATG, VB, CTK
ESCRT-III related *Regulation of membrane scission and ESCRT-III disassembly*	Vps60	CHMP5	Vta1	MVB, CTK, VB
	Vps46/DID2	CHMP1A,B	CHMP1, VPS4, LIP5, UBPY, AMSH, Spastin	MVB, CTK
	Ist1	IST1	VPS4, LIP5, CHMP1and VPS37(in mammals)	MVB, CTK
	-	CHMP7	CHMP4B, UBPY	MVB, VB
Vps4 and Vta1 *ESCRT-III disassembly*	Vps4	VPS4A,B (SKD1)	VPS4, LIP5, IST1, CHMP1, CHMP2, CHMP6	MVB, ATG, VB, CTK
	Vta1	LIP5	MIMs of ESCRT-III, VPS4	MVB, ATG, VB, CTK
Others *Modulatory and targeting roles*	Vps31/Bro1	Alix/HD-PTP	CHMP4, AMSH, UBPY, CEP55, viral YPXL motifs.	MVB, VB, CTK

* For the interactions, when the mammalian orthologue is indicated it means that in the yeast system both proteins also interact. If only the yeast protein is indicated, the human interaction has not been reported.

** MVB: MVB biogenesis; ATG: autophagy; VB: viral budding; CTK: cytokinesis.

Mammalian ESCRT machinery consists of six biochemically distinct multi-subunits sub-complexes termed ESCRT-0, I, -II, and –III, ALIX (also known as BRO1) and vacuolar protein sorting- associated 4 VPS4–LIP5 (Babst et al. 2002a; Babst et al. 2002b; Wollert and Hurley 2010).

The ESCRT machinery is able to pinch off membranes necks and release the given vesicle products with different sizes. Classical clathrin-coated membrane budding events give rise to fixed vesicle diameters while the size of the vesicles formed by the ESCRT pathway is heterogeneous. Moreover, ESCRT are also implicated in processes implying membrane remodeling other than endocytosis such as wound repair, cytokinesis or virus budding beside others (see *section 3.3 ESCRT functions*).

After more than a decade of research, the biomechanics of ESCRT assembly and the catalysis of membrane budding and scission have been fairly well described.

Important advances have been reached recently from electron microscopy analysis of membranes tubes formed by over-expression of ESCRT-III subunits either *in vivo* or using in *vitro* made giant unilamellar vesicles (GUVs) (Hanson et al. 2008; Lata et al. 2008a; Bajorek et al. 2009a; Bajorek et al. 2009b; Im et al. 2009; Wollert et al. 2009a; Wollert and Hurley 2010; Tang et al. 2016; Mierzwa et al. 2017; Schoneberg et al. 2017). However, one of the most challenging current goal of the field is to understand how the ESCRT-III complexes assembly on endosomal membranes and how they are differentially regulated in each of the processes in which they are involved. Moreover ESCRTs dysfunction contributes to diseases ranging from neurodegeneration to cancer especially due to their role in regulating membrane receptors and cytokinesis (Raiborg and Stenmark 2009; Stuffers et al. 2009; Mattissek and Teis 2014).

3.2 Evolution and Conservation

Phylogenetic studies place the appearance of ESCRTs encoding genes during the early eukaryotic evolution (Field et al. 2007). The ESCRTs first emerged in the *Archaea*, and their earliest function of these proteins was in cell division (Lindas et al. 2008; Samson et al. 2008). The ESCRTs emergence is contemporary to the origin of the endomembrane system and all the machinery necessary for its regulation (Schlacht et al. 2014).

Although ESCRT-I and ESCRT-II are nearly as ancient as ESCRT-III and VPS4, they were firstly found in the genomes of the *Lokiarchaeota*, a later evolved phylum of archaea-bacteria with eukaryote-like membrane trafficking processes, but not in many other archaea phylum (Spang et al. 2015). However, the Vps4 ATPase and the ECSRT-III complexes that participate in cell division, intracellular membrane remodeling and micro-vesicles formation (mammalian exosomes) were present already in all the archaeal ancestors, suggesting that the ESCRT-III proteins possess the primary biochemical function in membrane scission among the ESCRT machinery (Samson et al. 2008; Samson and Bell 2009; Makarova et al. 2010) whereas, the additional biochemical activities (like the recruitment of ubiquitinated membrane proteins) of the ESCRTs may be later elaborations (Samson et al. 2008; Samson and Bell 2009; Wideman et al. 2014; Hurley 2015).

The whole ESCRT machinery is well conserved in all eukaryotes, but its complexity has arisen during the evolution of multicellular organisms. Membrane remodeling functions appeared early in the evolution, whereas the role of the ESCRT machinery in controlling cell signaling, may have contributed early in the

evolution to the intercellular communication in multicellular organisms. Several duplications of ESCRT genes have been fixed during multicellular organism evolution, and new functions have been assigned to them (Michelet et al. 2010).

Concerning the ESCRT-III machinery, it is worthy to discuss that its emergence correlates with the appearance of the ubiquitin system in archaea (Grau-Bove et al. 2015). ESCRT-III polymerization is intrinsically necessary for membrane remodeling functions, however, there is no ESCRT-0, I or II in the *archaea* ancestor, suggesting that the polymerization of the ESCRT-III originally can occur independently of nucleation or interaction with those systems (i.e. ESCRT other than ESCRT-III). Indeed, *in vitro* analyses of ESCRT-III polymerization have shown that this process can happen spontaneously without the necessity of interaction with other ESCRT members or with lipidic membranes. However, very high non-physiological concentrations were used to observe this spontaneous polymerization (see section 3.4.3.3 Dimerization and Oligomerization models).

3.3 Functions of the ESCRT Machinery

The ESCRT machinery catalyzes deformation and reverse topology scission of lipidic membranes, from the concave side of the deformation in most of the cases. Many cellular processes are ESCRT dependent (Fig. 13):

-The formation of ILVs at the multivesicular bodies (MVBs) in the endo-lysosomal pathway (Hanson and Cashikar 2012) (See details below).

-The budding of enveloped viruses like the HIV (Garrus et al. 2001; Martin-Serrano et al. 2001; VerPlank et al. 2001; Martin-Serrano and Neil 2011; Effantin et al. 2013; Votteler and Sundquist 2013; Weissenhorn et al. 2013; Abdul Rehman et al. 2016).

-The mid-body abscission during cytokinesis (Carlton and Martin-Serrano 2007; Carlton et al. 2012; Caballe et al. 2015).

-The formation and release of micro-vesicles and exosomes (Baietti et al. 2012; Matusek et al. 2014; Olmos and Carlton 2016).

-The plasma membrane wound repair (Jimenez et al. 2014).

-The neuron pruning (Loncle et al. 2015).

-The EBV exit from the nucleus by budding through the nuclear envelope (Lee et al. 2012).

-The formation of viral replication compartments (Diaz et al. 2015).

-The NPC integrity and quality control (Webster and Lusk 2015)

-The micro-autophagy (Sahu et al. 2011).

The nature and the sequence of ESCRT recruitment in these different cellular processes of membrane deformation and scission are slightly different (Fig. 14). MVB biogenesis, cytokinesis, HIV budding and release, and autophagy are considered the canonical ESCRT pathways.

For the interest of this study, I will focus on the mechanistic description of the ESCRT machinery function during MVB biogenesis. Some aspects of their function in cytokinesis, virus budding and autophagy are only shortly described below.

Cytokinesis: this term defines the final step of scission of the membrane of two dividing cells that leads to their separation. During cytokinesis, the membrane necks between the two dividing cells surrounds the microtubule central spindle, where the centrosomal protein CEP55 is placed. CEP55 recruits ESCRT-I and Alix which recruits ESCRT-III. However the spindle needs to be cleaved before the scission can take place. By interaction with the ESCRT-III member CHMP1B, the microtubule-cutting enzyme Spastin is recruited and the scission is induced (Yang et al. 2008; Connell et al. 2009).

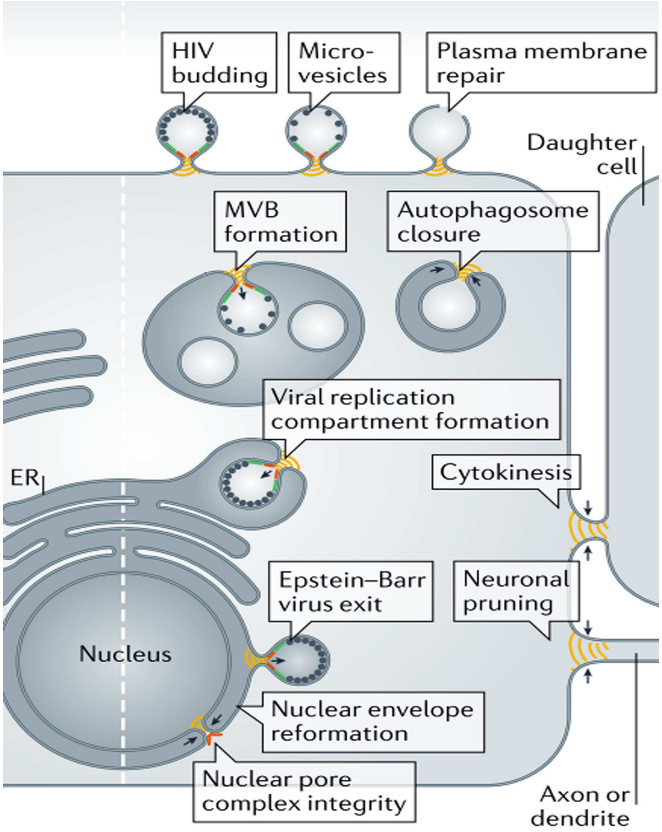


Figure 13: Functions of the ESCRT-mediated reverse-topology scission pathway (Schoneberg et al. 2017).

HIV budding and release: Membrane enveloped viruses exit the infected cell by an ESCRT dependent budding process. The most intensively studied is the viral budding pathway used by the HIV (Human immunodeficiency virus) in which ESCRT-I, Alix, ESCRT-III and VPS4 are recruited to nascent virions. ESCRT-I recruitment and bud formation that normally depends on the function of ESCRT-0 and II are ensured by the HIV protein Gag (Langelier et al. 2006; Effantin et al. 2013; Weissenhorn et al. 2013).

Importantly both, **cytokinesis and HIV budding** are thus independent of ESCRT-0 and II. The puzzling point here is which proteins or events other than ESCRT-II are mediating the activation and the recruitment of the ESCRT-III. Obviously, in an endomembrane containing cell, the membrane concavity or convexity cannot trigger a polymeric reaction by itself without other levels of regulation. Whatever the mechanism, it may be conserved from the *archaea* until the humans.

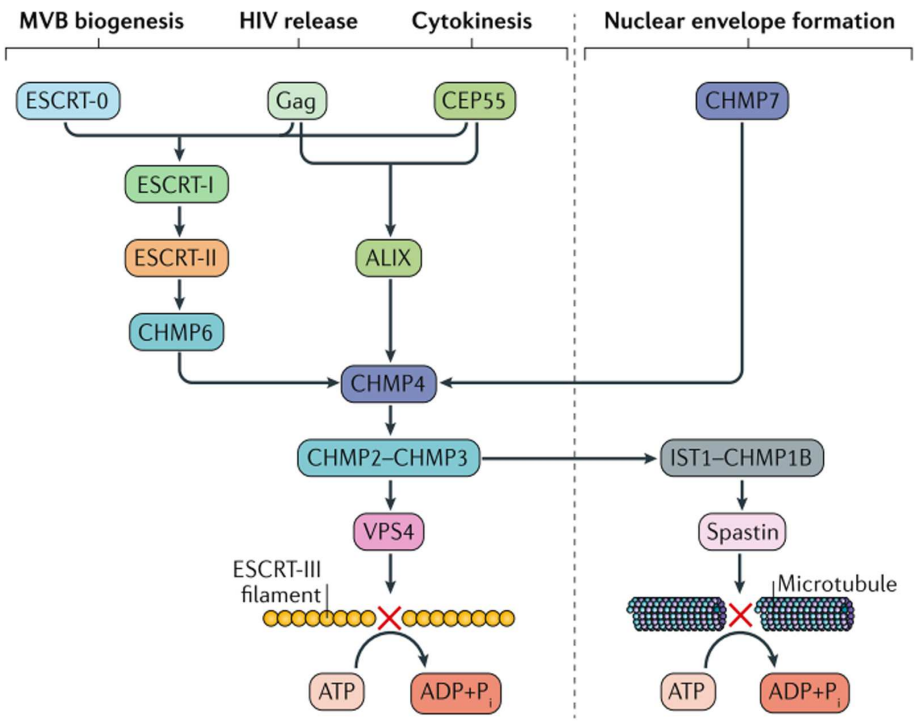


Figure 14: Schema of the three branches of the ESCRT pathway (Schoneberg et al. 2017).

Autophagy: all the autophagy processes require the formation of a phagophore. Phagophore is a double membrane structure that will elongate to form a double membrane vesicle. Therefore the closure of the phagophore is topologically identical to the ILV scission into the MVB. The ESCRT machinery is necessary for phagophore closure, however, this role may be at least partly indirectly due to the necessity of a intact endocytic pathway for the proper functioning of the autophagy pathways (Rusten and Stenmark 2009; Jacomin et al. 2016).

Moreover, the ESCRT machinery has essential roles in the immune response, for the antigen presentation in phagocytic cells, as well as in the development of multicellular organisms. The ESCRTs notably regulate developmental signals through the control of long range cell-cell communication, a complex process dependent on the production of signals or morphogens and in the interpretation of their gradients (see also *chapter 5. Endocytosis and Developmental Signals*).

Endocytic control of receptors and signaling will be described later on in the *chapter 4: Ubiquitin System in Endocytosis of Membrane Receptors* with special focus on the EGF and Notch signaling pathways. Also reviewed in (Gonzalez-Gaitan 2003a; Gonzalez-Gaitan 2003b; Gonzalez-Gaitan and Stenmark 2003; Gonzalez-Gaitan

2008; Sorkin and Goh 2008; Furthauer and Gonzalez-Gaitan 2009; Merrifield and Kaksonen 2014).

3.4 The ESCRT Machinery in MVB Biogenesis

3.4.1 Introduction

ESCRT proteins have a dual role in MVB biogenesis: (1) they mediate the sequestration of cargoes at the endosomal membrane, and (2) they induce budding and scission of the endosome membrane away from the cytosol to form ILVs loaded with the sequestered cargoes.

Well known cargoes whose trafficking and sorting are regulated by the ESCRT pathway are: RTKs , particularly the EGFR (Babst et al. 2002a; Lu et al. 2003; Orth et al. 2006; Berlin et al. 2010; Goh et al. 2010), the PVR (Poliovirus Receptor) and Torso (Lloyd et al. 2002; Jekely and Rorth 2003); non-RTKs such as Notch, Dpp and HH (Jekely and Rorth 2003) ; the TGF-β receptor (Shim et al. 2006) , the E-cadherin (Toyoshima et al. 2007), the IL-2 receptor (Yamashita et al. 2008), gap junction proteins, ion channels, transporters and GPCRs (!!! INVALID CITATION !!!). In plants, the ESCRT machinery regulate Auxins transporters called PIN proteins (Spitzer et al. 2009).

Studies with purified ESCRTs (from bacterial expression system) and GUVs have suggested a sequential order of ESCRT functions in ILVs formation (Teis et al. 2008; Wollert and Hurley 2010). Several architectures models have been actually proposed for the sequential ESCRT machinery assembly, however, the linearity of ESCRT recruitment is presently in question (see *section 3.4.3 ESCRT-III/CHMP Proteins: Membrane Scission*).

Moreover, local asymmetry in lipid composition may have an important role in the ILVs formation facilitating the invagination of special lipidic micro-domains (Kobayashi et al. 1998; Wurmser and Emr 1998).

3.4.2 ESCRT-0, I and II: Receptor Recognition, Clustering and Budding

Each of the three early ESCRT complexes (ESCRT-0, -I, and -II) can interact with ubiquitinated cargoes with low affinity. Probably, they cooperate to sort ubiquitinated cargoes to the MVB pathway. When cargoes are clustered, ESCRT-I

and ESCRT-II can initiate the budding of the endosomal membrane. Comforting this hypothesis, large buds have been observed *in vitro* following the recruitment of ESCRT-I and ESCRT-II together to membrane surface at physiological concentration (Saksena et al. 2009; Wollert et al. 2009a; Wollert and Hurley 2010). Of note, the yeast Vps20 and Snf7 subunits of the ESCRT-III are also able to generate buds *in vitro* although not at physiological concentrations.

ESCRT-0:

The first step of the ESCRT activity in receptor sorting is the binding of the FYVE domain of the ESCRT-0 protein Vps27/HRS to the endosomal membrane through the lipid phosphatidylinositol 3-phosphate (PI3P). HRS together with the other ESCRT-0 subunit Hse1/STAM and accessory proteins like Eps15B bind both the ubiquitinated cargoes with its UBD and clathrin (Raiborg et al. 2001a; Raiborg et al. 2001b; Raiborg et al. 2002; Bache et al. 2003a; Bache et al. 2003b). This results in the clustering of the cargoes in lipidic micro-domains. Both HRS and STAM contain SH3 domains that can recruit the two DUBs: UBPY and AMSH (Kato et al. 2000; McCullough et al. 2006; Sierra et al. 2010). These interactions mediate the deubiquitination of the cargoes to send it out of the degradative pathway or trigger the inactivation of components of the machinery that are ubiquitinated (Kato et al. 2000; Clague and Urbe 2006; Alwan and van Leeuwen 2007; Clague et al. 2012a; Clague et al. 2012b; Zhang et al. 2014; Pradhan-Sundd and Verheyen 2015)(see *section 3.5 Post-translational Regulation of the ESCRT Machinery*).

ESCRT-I:

The PSAP (prosaposin) domain of HRS binds to and therefore recruits the ESCRT-I subunit TSG101 (Fig. 15). ESCRT-I is a stable hetero-tetramer formed by Vps23/TSG101, Vps28, Vps37 and Mvb12 (also named ubiquitin-associated protein 1 (UBAP1). TSG101 contains an ubiquitin E2 variant domain (Kuz'mina et al.) that binds the ESCRT-0 HRS and ubiquitinated cargoes (Katzmann et al. 2001; Katzmann et al. 2003; Amit et al. 2004). Vps37 is involved in interaction with the lipidic membrane. The C-terminal domain of Vps28 recruits ESCRT-II (Strack et al. 2003). Therefore ESCRT-I is implicated in the initial membrane invagination and clustering of ubiquitinated cargoes and in the recruitment of ESCRT-II, or in some cases directly of ESCRT-III, proteins.

ESCRT-II:

The ESCRT-II forms a nexus between ubiquitinated cargoes, the endosomal membrane and the ESCRT-I and ESCRT-III complexes. It is composed of Vps36/EAP45 and Vps22/EAP22 and two Vps25/EAP20 molecules forming a Y-

shaped stable hetero-tetramer. The GLUE domain of Vps36 interacts with Vps28 of the ESCRT-I and also with PI3P and ubiquitin. Vps22 interacts with the lipidic membrane. The complex consisting of ESCRT-I and –II has the capacity (at least in a GUV model) to induce membrane invaginations (Bache et al. 2003b; Teo et al. 2004; Kostelansky et al. 2006; Wollert and Hurley 2010). Therefore it has been proposed a model in which ESCRT-0 complex clusters the cargoes and the ESCRT-I-II complex buds the membranes (Teis et al. 2008; Teis et al. 2010). The C-terminal domain of Vps25 binds to the ESCRT-III subunit CHMP6. The Y-shape of ESCRT-II results in two CHMP6 binding sites at two of the three branches of the Y. Thus, it is proposed that ESCRT-II and the two CHMP6 proteins form a curved complex that putatively contribute to the nucleation of ESCRT-III filaments (Fig. 15) (Hierro et al. 2004; Yorikawa et al. 2005; Pineda-Molina et al. 2006; Im et al. 2009; Teis et al. 2010; Christ et al. 2016).

Bro1/Alix:

ALIX was initially isolated based on its association with an apoptosis-related protein, ALG-2. In yeast, Bro1 is not strictly required for MVB biogenesis and is mainly involved in the recruitment and activity of the deubiquitinating enzyme Doa4 to the assembling ESCRT-III complex (Luhtala and Odorizzi 2004). ALIX also has a central role in targeting the ESCRT machinery to the midbody for membrane abscission in cytokinesis and is essential for the budding of certain viruses (Missotten et al. 1999). The particularity of Alix is that it interacts with the ESCRT-I TSG101 and bridges it directly to the ESCRT-III CHMP4 (von Schwedler et al. 2003) and that it can sense negative curvature in invaginating membranes (Matsuo et al. 2004).

3.4.3 ESCRT-III/CHMPs: Membrane Scission

The ESCRT-III complex is a dynamic hetero-polymer, without a clear defined composition to date, that only transiently assembles on endosomes.

The main function of the ESCRT-III proteins is to catalyze membrane scission. The energy for the reaction comes from inter-subunit and subunit-membrane interaction formed during the assembly of the complex in/on the membranes and also most probably from the hydrolysis of ATP needed for its disassembly (see the proposed models in the *sections 3.4.3.5 Membrane Remodeling and Scission by CHMP Proteins* and 3.4.3.6 Disassembly of the ESCRT-III Polymers: Vps4).

The ESCRT-III (and ESCRT-III related) family includes IST1 and the Charged Multivesicular Proteins (CHMPs): CHMP1A and CHMP1B (also known as Did2 in yeast); CHMP2A and CHMP2B (also known as Vps2 in yeast); CHMP3 (also known as Vps24 in yeast); CHMP4A, CHMP4B and CHMP4C (also known as Snf7 in yeast); CHMP5 (also known as Vps60 in yeast); CHMP6 (also known as Vps20) in yeast); and CHMP7 (also known as Chm7 in yeast). For the formation of MVB, only Vps20 and Snf7 are absolutely required while to date, loss of function of the other CHMPs failed to induce significant phenotype, putatively due to functional redundancy (Babst et al. 2002b; Babst 2011) (See Table 1).

CHMPs are a family of strongly conserved proteins, of relatively small size of 221–241 amino acids, with similar structure and biochemical properties. The first 100 N-terminal residues of these proteins form helices α1–α2 which are basic and electropositive and are structured in a rigid 2-helix hairpin of 70Å important for membrane binding and homo- or hetero-dimerization. The helices α3 to α6 are shorter and electronegative, and the C-terminal helices α5 and α6 have regulatory roles. The four N-terminus helices α1 to α4 are the main structural components of ESCRT polymers and they form an antiparallel four-helix bundle. Both terminus contain predicted coiled-coils (Fig. 16) (Muziol et al. 2006; Zamborlini et al. 2006; Shim et al. 2007; Lata et al. 2008a; Bajorek et al. 2009b; Lata et al. 2009). All CHMPs contain MIM domains of different subtypes at their C-terminal region that interact with MIT domain containing proteins (Table 2) (Solomons et al. 2011).

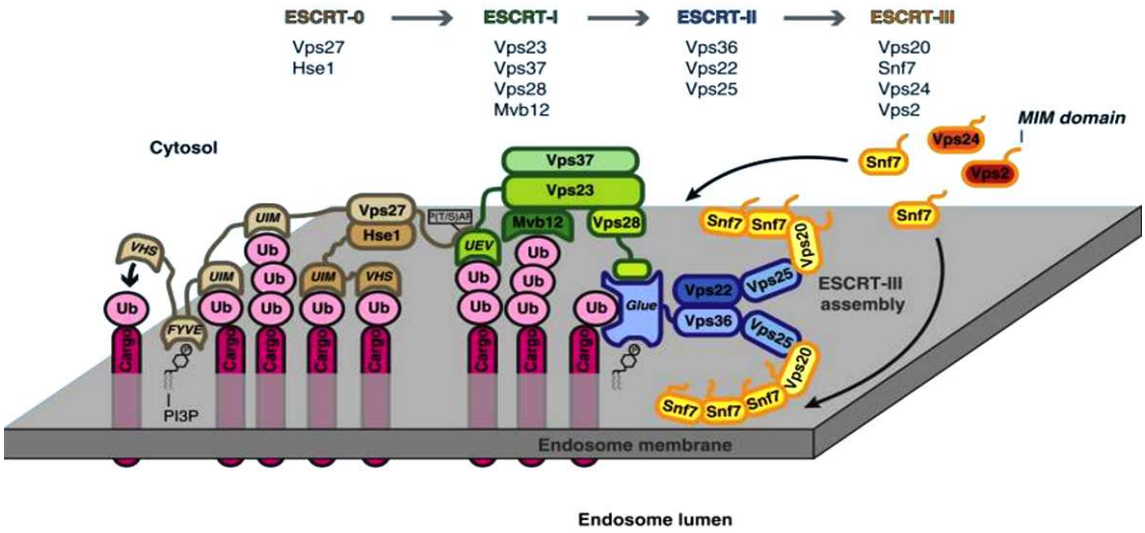


Figure 15: Model for cargoes clustering and sequential assembly of the ESCRT -0, I, and III complexes in yeast. Modified from (Schmidt and Teis 2012).

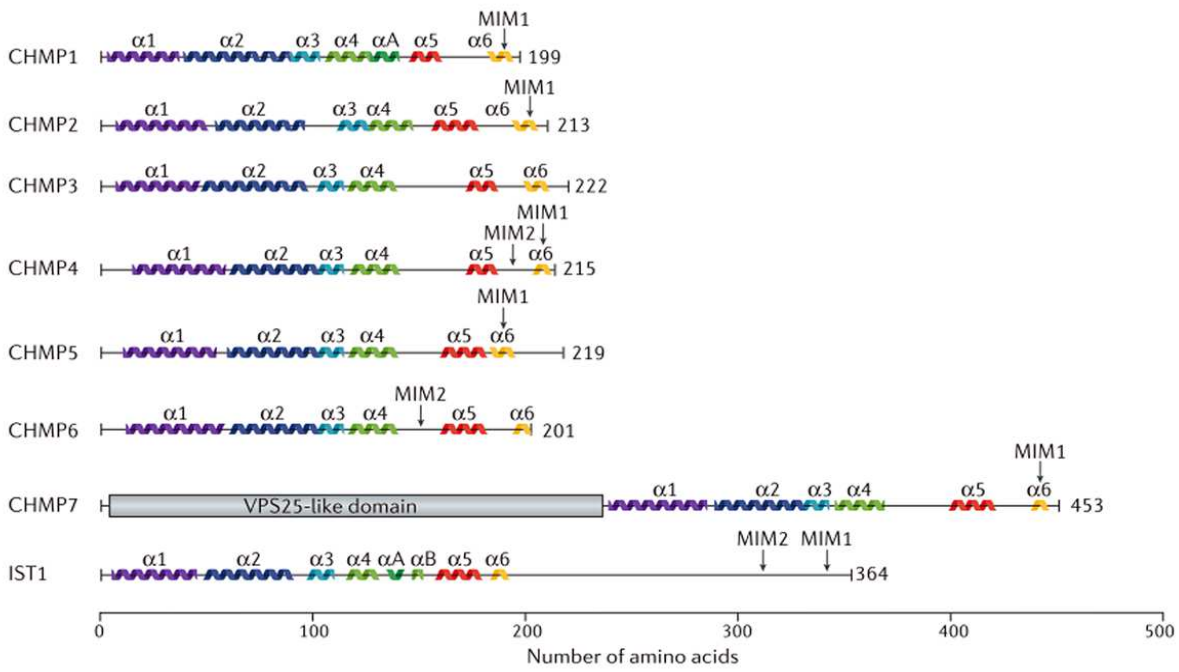


Figure 16: Primary structural organization of the 12 human ESCRT-III proteins. Yeast Snf7, CHMP2B and CHMP1B are shown as representatives of the CHMP4A–CHMP4C, CHMP2A–CHMP2B and CHMP1A–CHMP1B families, respectively. The core structural helices are α1, α2, α3, α4, αA and αB. The α5 is an auto-inhibitory helix and α6 is an interaction helix. The MIM1 and/or MIM2 are responsible for binding to MIT containing proteins. CHMP7 is a hybrid ESCRT-II-like and ESCRT-III protein, with its amino-terminal domain resembling the ESCRT-II subunit VPS25. Modified from (Schoneberg et al. 2017).

Table 2: ESCRT-III MIM domains

ESCRT-III	MIM domain	Interactor	Publication
CHMP1A	MIM1	VPS4, IST1,	(Obita et al. 2007; Stuchell-Brereton et al. 2007)
CHMP1B	MIM1, MIM3	VPS4, IST1, Spastin, UBPY	(Obita et al. 2007; Stuchell-Brereton et al. 2007; Yang et al. 2008)
CHMP2A,B	MIM1	VPS4	
CHMP3	MIM1, MIM4	AMSH	(Solomons et al. 2011)
CHMP4A-C	MIM1, MIM2	Alix, VPS4	(Kieffer et al. 2008)
CHMP5	MIM1	LIP5	(Skalicky et al. 2012)
CHMP6	MIM2	VPS4	(Kieffer et al. 2008)
CHMP7	MIM1, MIM2		(Vietri et al. 2015; Christ et al. 2017)
Ist1	MIM1, MIM2		(Schoneberg et al. 2017)

3.4.3.1 CHMPs Conformations: Auto-inhibition and Activation

Most of the knowledge on CHMPs molecular structure come from *in vitro* studies and the high-resolution structures available for CHMP3 (Muziol et al. 2006), CHMP4 (Tang et al. 2015), IST1 and CHMP1B (McCullough et al. 2015).

These studies indicate that CHMPs exist in two states (Fig. 17):

The inactive state is a monomeric cytoplasmic state where α5 and α6 fold against the core α1–α4 to prevent higher order assemblies. This state is also referred to as closed conformation or auto-inhibited conformation (Lin et al. 2005; Muziol et al. 2006; Shim et al. 2006; Zamborlini et al. 2006).

In the active state, also referred to as open conformation, the region of the α1–α4 core is exposed and accessible for interaction with other CHMPs and membranes. Polymerization in *vivo* is believed to only happen in association with the lipidic membranes. However, *in vitro*, truncations of the inhibitory helix α5 of various CHMPs or increasing the salt concentration of the buffer solution is sufficient to induce their assembly into filaments even in the absence of membranes, probably by exposing the dimerization region of the α1–α4 core (Babst et al. 2002a; Babst et al. 2002b; von Schwedler et al. 2003; Tsang et al. 2006; Zamborlini et al. 2006; Shim et al. 2007; Tang et al. 2016).

The activation of the CHMPs requires important intramolecular conformational changes that relieve auto-inhibition and enable interaction with other ESCRT-III molecules and membranes, also resulting in exposure of the MIM domains. In the case of CHMP4 or CHMP1B, helices α2 and α3 are melded into a single helix extending the hairpin α1-2. Thus, regions that are unstructured in the closed conformation become rigid and join helix α4 to extend it in the open conformation (Fig. 17) (McCullough et al. 2015; Tang et al. 2015).

In the particular case of CHMP1B, it remains monomeric even under high ionic strength conditions. Decreasing the ionic strength induces the opening of the molecule by displacing of ~100 Å the helix α5 hand (Fig. 17).

However, although the intramolecular conformational changes might be very similar between CHMPs, the inducer(s) of this conformational change are still not well established and possibly depend on the type of CHMP and the specific process in which it is implied at a given moment.

Moreover, the ESCRT-III subunit IST1 is the only case shown to polymerize *in vitro* in the closed conformation (McCullough et al. 2015), whereas other ESCRT-III

polymerize only in the open conformation (see *section 3.4.3.3 Dimerization and Oligomerization Models*).

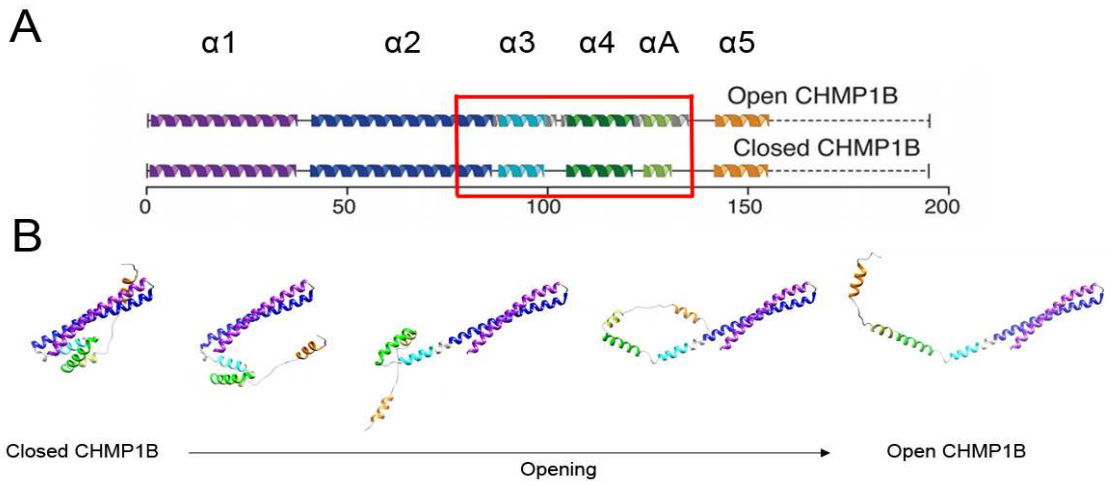


Figure 17: Structure and conformational changes proposed for CHMP1B. A: Secondary structure diagrams for open CHMP1B (middle), and closed CHMP1B (bottom). **B:** Ribbon model of CHMP1B monomers from closed to open. The rectangle shows the fusion of the helixes α2 to α3 and the elongation α4 when CHMP1B opens. Modified from (McCullough et al. 2015).

3.4.3.2 Nucleation, Recruitment and Activation

ESCRT-III proteins carry out their function by assembly in association with lipidic membranes, therefore necessitating a recruiting step.

Classically, the recruitment point is believed to be the association of Vps20/CHMP6 to the ESCRT-II and the lipidic membranes as shown in both mammalian cells and yeast (Yorikawa et al. 2005). However, three subunits of the yeast ESCRT-III: Vps20, Snf7 and Vps24, are sufficient to detach ILVs from GUVs (Wollert et al. 2009a), although this is not true at physiological concentrations (Im et al. 2009). *In vitro,* at physiological concentration, the binding of the α1 of the yeast Vps20 to Vps25 it is proposed to displace α5 and α6 releasing Vps20 auto-inhibition and inducing its binding on GUVs and subsequent membrane deformation (Teo et al. 2004; Teis et al. 2008; Im et al. 2009; Saksena et al. 2009; Wollert et al. 2009a; Wollert et al. 2009b; Teis et al. 2010). Therefore, Vps20/CHMP6 interaction with Vps25/EAP20 is still considered as the activation and nucleation point for the ESCRT-III polymerization reaction (Fig. 15).

In yeast, the Y shaped ESCRT-II implying two copies of Vps25 can nucleate functional ESCRT-III complexes during MVB biogenesis by the initiation of two filaments, while a single Vps25 can activate Vps20 but induce the formation of non-functional Snf7 oligomers (Teis et al. 2010) (Fig. 15). It has been proposed also that the high protein concentration at ILVs may trigger the opening and activation of Vps20/CHMP6 (Teis et al. 2008; Teis et al. 2010).

Once activated, Vps25/CHMP6 can interact with Snf7/CHMP4 which is the major ESCRT-III component of the endosomes and recruits other ESCRT-III subunits (Fig. 15) (Saksena et al. 2009; Wollert et al. 2009a; Wollert and Hurley 2010; Henne et al. 2012). The presence of negative membrane curvatures accelerate nucleation *in vitro* although it is not sufficient to initiate polymer formation (Teo et al. 2004; Saksena et al. 2009; Lee et al. 2015). Recruitment and activation of ESCRT-III is proposed as the control mechanism for specific and temporal regulation of cargoes sequestration during MVB (Teis et al. 2010).

In yeast two-hybrid system, a triangular network between ESCRT-I, -II and –III has been revealed, questioning the strict linearity of interactions of these complexes (Bowers et al. 2004). Moreover, ESCRT-II or CHMP6 are not necessary for the recruitment of ESCRT-III in cytokinesis and virus budding processes (Pineda-Molina et al. 2006). Bypassing ESCRT-II is also possible when CHMP4B is recruited by Alix; however, it is not clear whether Alix activates CHMP4B, increases its local concentration or just stabilizes the CHMP4B filament (Pires et al. 2009; Henne et al. 2011; Tang et al. 2016). In addition, during the viral budding, CHMP6 is not even implicated (Martin-Serrano and Neil 2011). Finally, there is neither ESCRT-I nor II nor Alix in *archaea* which possess ESCRT-III proteins and Vps4. All these features indicate that in some circumstances, ESCRT-III must trigger membrane scission by themselves. However, scission of ILVs on GUVs using only this proteins at physiological concentration has not been yet achieved (Wollert et al. 2009a).

Most of the IST1 and CHMPs interactions with other ESCRT-III and/or with partners other than ESCRT important for the activation of the CHMP proteins map to the C-terminal region. For example, Did2 (orthologue of CHMP1) interacts with itself and with Vps4, Vta1 or the enzymes Spastin, UBPY and AMSH; but not with other ESCRT-III proteins. In the case of the human CHMP3 it has been suggested that interaction with AMSH induces the activation and membrane recruitment of CHMP3 (Zamborlini et al. 2006; Lata et al. 2008a).

3.4.3.3 Dimerization and Oligomerization Models

All the dimerization or oligomerization models and interaction interfaces proposed for CHMP proteins come from *in vitro* studies which *in vivo* significance has been still poorly tested. Different models have been proposed for different CHMP proteins:

CHMP3 or CHMP2/3 model

In solution, purified full-length CHMP3 adopts a closed metastable conformation in low ionic strength solution and an open conformation in high ionic strength solution (Lata et al. 2009). The activation of the molecule can be achieved also by mutating the regulatory C-terminal region.

From the crystal structure of C-terminal CHMP3 (residues 9-183) an antiparallel dimer has been observed and two different interfaces have been described to be necessary for its formation (Fig. 18):

(1) **A "side-to-side antiparallel dimer"** interface has been postulated as a possible ESCRT-III assembly mode. In this model, the dimerization is mediated by α2-α2 interactions with minor contributions from α1 and α5. The position of the α5 is determined by the antiparallel dimeric position of the core. This dimer has a large flat basic surface which has been identified to be necessary for membrane targeting *in vivo* and for HIV-1 budding. K49 residue of CHMP3 is exposed on this surface and is implicated in the binding to PtdIns(3,5)P2 (Whitley et al. 2003). Moreover, this kind of dimeric membrane interaction permits the access of VPS4 to the point of scission which is required for final disassembly of the complexes (Lin et al. 2005; Yorikawa et al. 2005; Muziol et al. 2006; Williams and Urbe 2007; Ghazi-Tabatabai et al. 2008; Lata et al. 2008a; Ghazi-Tabatabai et al. 2009; Lata et al. 2009). Polymerization of ESCRT-III is a concomitant process to their recruitment to membrane and dimerization mutants are actually not able to localize in the membranes (Muziol et al. 2006; Lata et al. 2008a). The opening of CHMP3 leads to its membrane binding and the formation of a detergent-resistance polymer of CHMP3 (Lin et al. 2005; Muziol et al. 2006; Shim et al. 2007).

(2) The second dimerization interface proposed for CHMP3 corresponds to a **"tip-to-tip dimer interface**" formed by the exposed hydrophobic surface located in the tip of the α1/2 loop which is another preferred site for protein-protein interaction (Muziol et al. 2006; Williams and Urbe 2007; Bajorek et al. 2009b; Tang et al. 2015)(Fig. 18).

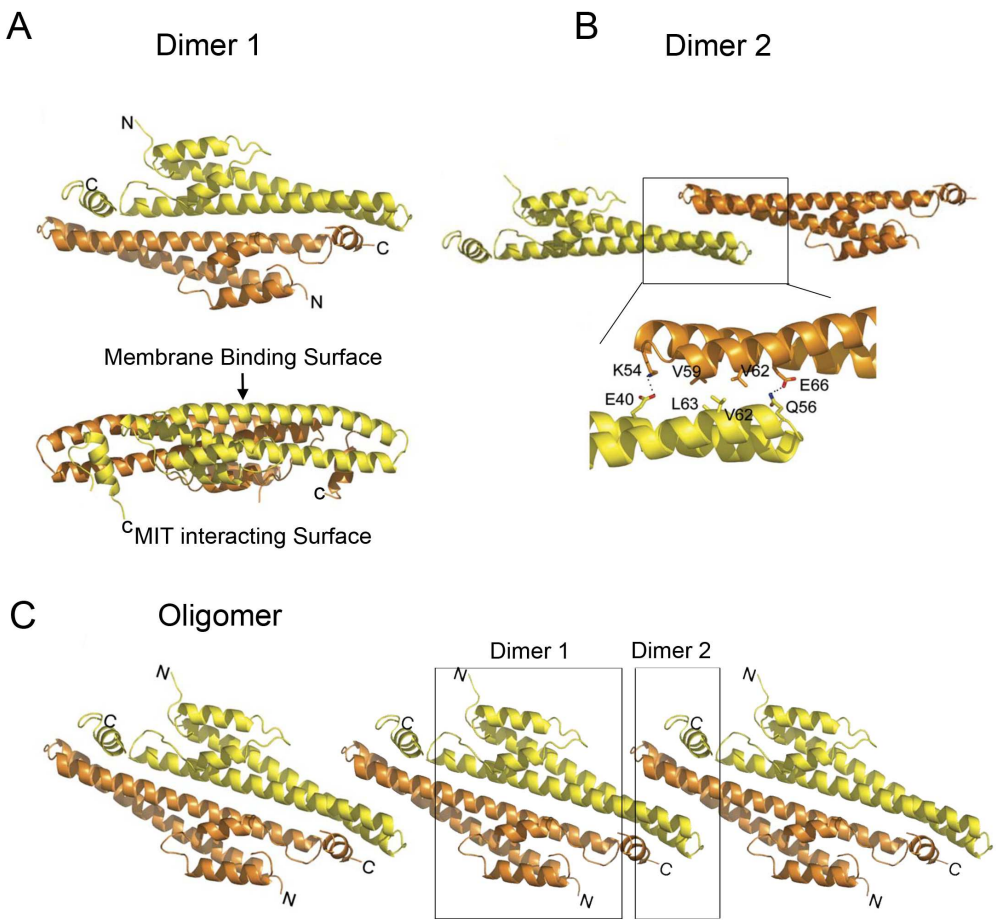


Figure 18: Model for CHMP3 oligomerization: A: Ribbon models of a CHMP3 9-183 dimer 1 from the front view and lateral view on the bottom. **B:** Ribbon models of a CHMP3 9-183 dimer 2 with the details of the interacting residues on the bottom. **C:** situation of dimer 1 and dimer 2 into the oligomers. Modified from (Muziol et al. 2006).

Yeast Snf7/Human CHMP4 model

Tang and colleagues have presented two X-ray crystal structures that unravel the molecular mechanism of Snf7 conformational activation and polymer assembly (Tang et al. 2015). Upon opening, the core domain undergoes a large-scale conformational rearrangement to extend into a highly elongated structure with α2-3 melded and which adopts a ~30 A° periodic protofilaments of parallel subunits on membranes. The membrane interacting region of Snf7 was described in (Buchkovich et al. 2013). In the polymer presented by Tang and colleagues several conserved lysine residues in α2 and 3 are exposed in the same direction in the electrostatic membrane-binding regions on the outside of the super-helix coupling

polymerization to stable membrane association. Three interacting interfaces have been described in this model (Fig. 19):

(1) Side-to-side parallel interface where the α2/3 helix of protomer (x) interact with α3 of its neighboring promoter (x+1).

(2) Electrostatic interactions between α1 of protomer (x) and α2/3 of protomer (x+1)

(3) α4 interacts with the α1/2 hairpin of another protomer in trans on a neighboring protofilaments which stabilize inter-filamental interactions.

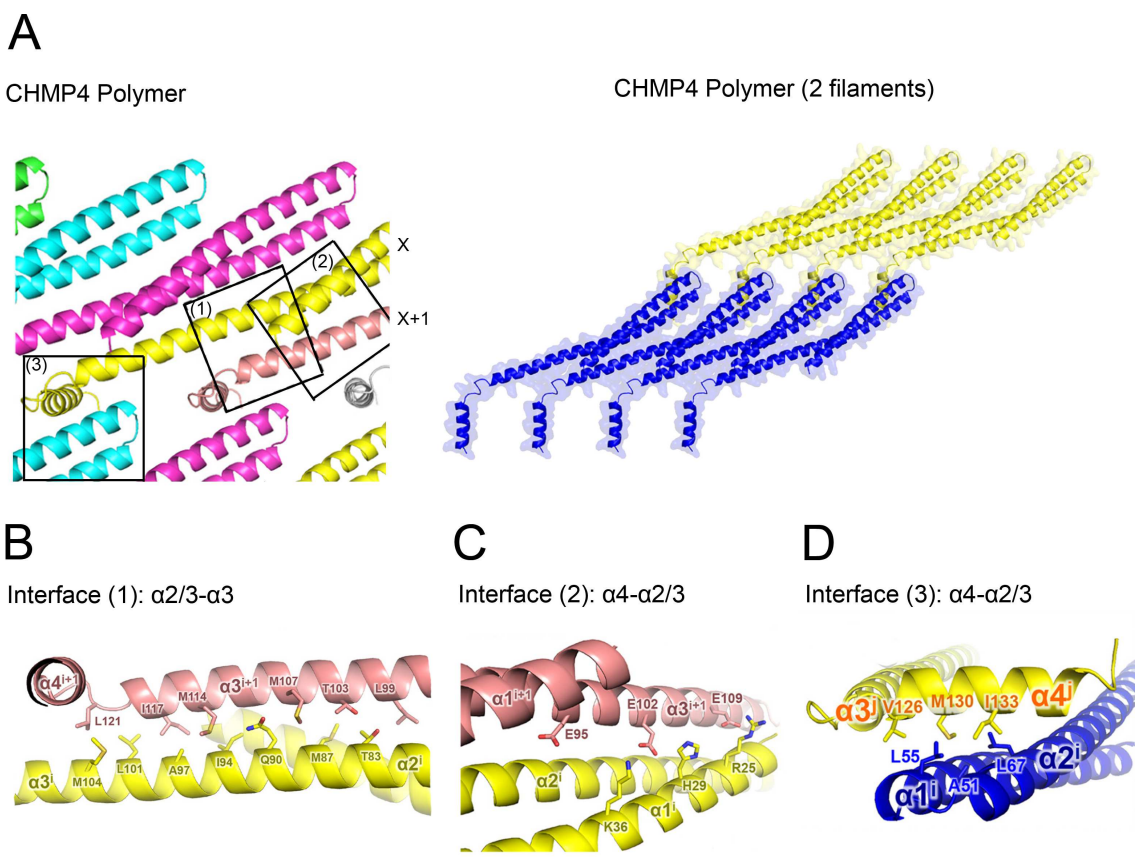


Figure 19: Model for Snf7/CHMP4 oligomerization: A: Ribbon models of a Snf7 filament showing the 3 different interfaces (left) and the two filaments polymeric structure (right). (1) The hydrophobic protein interface α2/3x and α3x+1, (2) the electrostatic interface between α1x and α2/3x+1, (3) hydrophobic interface between α4j (yellow) and α1/2 (blue) of another filament. **B-C-D:** Close-up views of (1), (2) and (3). Protomer (x) shown in yellow and protomer (x+1) in salmon.. Modified from (Tang et al. 2015).

Yeast Did2-Ist1 and human CHMP1B/IST1model

Did2 and Ist1 and their human orthologues CHM1B and IST1 are called ESCRT-III associated proteins since they have non-essential, but important roles in the MVB pathway.

Did2/CHMP1B: In 2001, in two back-to-back papers from Hollenberg and co-workers and Stauffer and coworkers, CHMP1B was identified respectively, as a cytoplasmic vesicle trafficking protein (Howard et al. 2001) and as a nuclear matrix protein (Stauffer et al. 2001). Given its ambiguous nature, it was named chromatin modifying protein/charged multivesicular body protein-1. While no further evidence has emerged illustrating the putative chromatin remodeling function of this protein, in yeast, the role of Did2 (homologous to CHMP4) in vesicle trafficking was confirmed as a modulator of the ESCRT-III dissociation from the endosomes by Vps4. Did2 is recruited to the endosomal membrane via the Vps24 and Vps2 subunits (Nickerson et al. 2006). This membrane localization is correlated with a conformational change leading to exposure of its MIM1 sequence (Bowers et al. 2004; Nickerson et al. 2006; Obita et al. 2007; Rue et al. 2008). Ist1 is then recruited to the endosomes by Did2 (Rue et al. 2008; Xiao et al. 2009) where it can interact with Vps4 and regulate its recruitment and activity. Cells lacking only Ist1 or Did2 present modest MVB cargoes sorting defects. Co-silencing of Ist1 with Vta1, Vps60, or Vps4, but not Did2, synthetically enhanced the phenotype. Therefore, the Ist1-Did2 complex can regulate Vps4 activity either in concert with the Vta1–Vps60 complex or, alternatively, both complexes may have redundant functions (Dimaano et al. 2008; Rue et al. 2008; Bajorek et al. 2009a; Xiao et al. 2009).

In human cells, the IST1-CHMP1B interaction is essential for cell abscission during cytokinesis as evidenced by the fact that IST1 mutants blocking CHMP1B binding, prevent cell abscission (Bajorek et al. 2009a; Xiao et al. 2009).

In yeast, Did2 is predominantly monomeric in the cytosolic fraction, while Did2-Ist1 complex is absent from the cytosol. Did2 is clearly necessary to directly or indirectly recruit Ist1 to the endosomes (Rue et al. 2008).

In vitro studies of the structure of polymeric IST1 alone or of CHMP1B alone have been reported:

IST1NTD Homo-polymers: EM analyses revealed that IST1 N-terminal domain (IST1NTD) assemblies in large tubular structures with diameters of 700 nm and open along one edge, like curled sheets (Bajorek et al. 2009a) or as closed tubes of

70 nm diameter (McCullough et al. 2015). Full length IST1 assembles in close conformation and both monofilament and 2 filaments tubular assembly were described (McCullough et al. 2015). The interaction interface IST1-IST1 in the case of the monofilament assembly is weakly described. In the case of the 2 filaments assembly, tip-to-tip interfilamentous interaction are required like in the case of CHMP3 (Bajorek et al. 2009b; McCullough et al. 2015) (Fig. 20A, B).

Did2/CHMP1B Homo-polymers**:** CHMP1B alone has the ability to assemble into helical tubes. CHMP1B tubes diameter may be of 230 nm (Bajorek et al. 2009a) or 90nm (McCullough et al. 2015) and are fully closed. Into the monofilament polymer, the subunits adopt the same much extended open conformation as described for CHMP4 (Fig. 20A, C). On lipidic membranes using large unilamellar vesicles (LUVs), CHMP1B can form single filament or double filament polymers (McCullough et al. 2015). The structure and functionality of the inter-filament interface has not been described, letting black boxes about the charges and curvature of the opposite surface facing out of the membrane.

Co-polymer Did2-Ist1 and CHMP1B-IST1: the MIM1 structure of CHMP1 had been first characterized in complex with two MIT domains of mammalian VPS4 (for MIM1 of CHMP1A) and spastin (for the MIM1 of CHMP1B) (Stuchell-Brereton et al. 2007; Yang et al. 2008) before characterization of the complex CHMP1B-IST1. In these two first examples, the MIT domain had a three-helix bundle structure α1, α2 and α3 and the interaction of the MIM domain occurred through a channel formed in association with either the α2 and α3 (VPS4) or the α1 and α3 (Spastin) of the MIT domain. However, in the case of IST1 the MIM-MIT interaction works differently. The first co-crystal structure of the purified stable complex of Did2-Ist1 was described in yeast in Xiao 2009 using Ist1NTD and the MIM1 domain of Did2. The MIM1 binds in a channel formed by three perpendicular helices of Ist1NTD (i.e., the α5 and α1/2 hairpin present in the closed conformation of Ist1). The molecular surface of the Did2-MIM1 domain that mediates association to the Vps4 and spastin MIT domains also participates in binding to Ist1 in this structure (Xiao et al. 2009; McCullough et al. 2015). (Fig. 21A). This structure implies that when α5 of Ist1 is in contact with the α1/2, i.e., in the "close conformation", the protein is nevertheless, in its active conformation, i.e.: in a conformation able to polymerize and interact with MIM1-Did2 but not with membranes. The loss of this interaction (α5 of Ist1 with its own α1/2 tip) leads to membrane binding independently of Did2 but the molecule is not functional in cytokinesis in *vivo* (Bajorek et al. 2009a). So, the closed conformation in interaction with Did2 was pronounced as the active form of Ist1 *in vivo*. The similarities between the Ist1 active-closed structure and the CHMP3

dimeric subunit leads to wonder if Ist1 can form the same kind of antiparallel dimers and sheet lattices as CHMP3. The human IST1 homo-polymers curled sheets published in (Bajorek et al. 2009a) leaves this possibility open but further studies are needed to decipher whether or not these dimer based polymeric structures may polymerize similarly as the CHMP3 dimers or the Amphiphysin2/BIN1 N-BAR protein (Muziol et al. 2006; Adam et al. 2015).

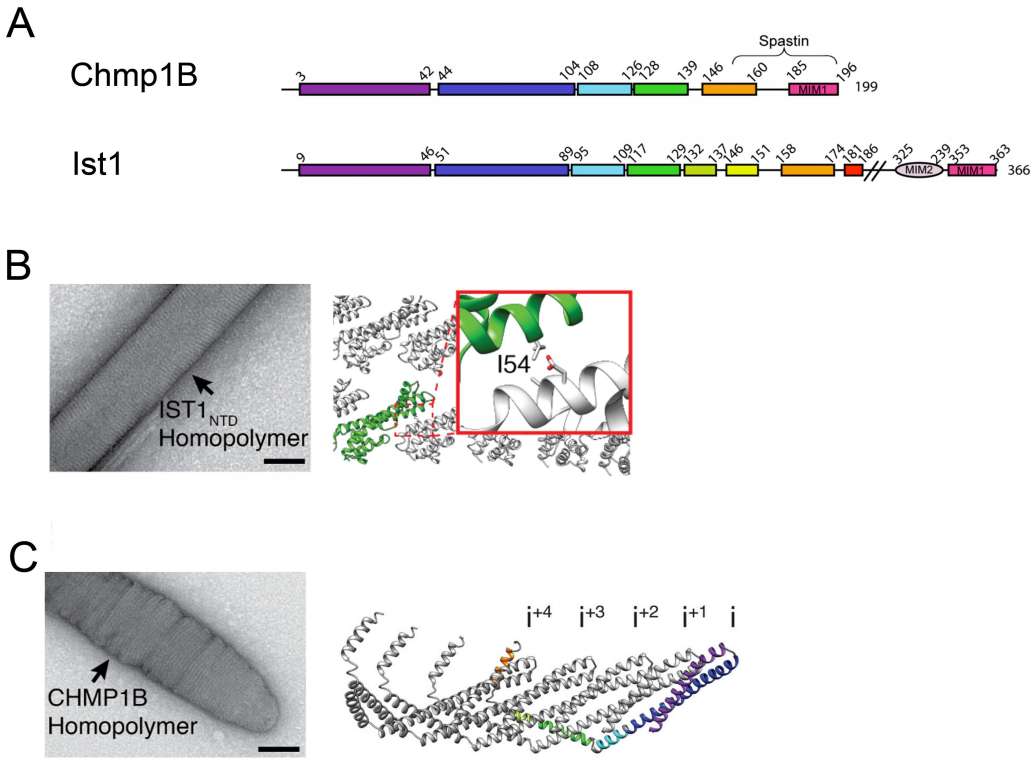


Figure 20: Model for Ist1 and CHMP1B homo-polymers: A: Secondary structural models of human ESCRT-III proteins. Helices are shown as blocks. Approximate residue boundaries for each helix are provided above each secondary structure. Vps4 interacting MIM1 domain and the binding site for the Spastin MIT domain on CHMP1B are also shown. **B:** Negatively stained electron micrographs of wild type IST1NTD homo-polymer (left). The interacting interface IST1NTD homo-polymers and the positions of mutated residue important for the interaction is shown (right); **C:** Negatively stained electron micrographs of wild type CHMP1B homo-polymers (left). Ribbon diagram of the modeled five interlocked CHMP1B molecules (right). Modified from (McCullough et al. 2015).

In yeast the MIM1 domain of Did2 cannot bind to Ist1 and Vps4 simultaneously *in vitro* (Xiao et al. 2009). How these two interactions are coordinated in the cells stayed intriguing until 2015 when McCullough and colleagues, using

either the full-length CHMP1B or the truncated CHMP1B1-175, showed that IST1 and CHMP1B spontaneously co-assembled under low ionic strength conditions into well-ordered helical tubes (Fig. 21B). In this study, they demonstrate that the interaction with IST1 was happening in the case of full-length CHMP1B only in a binary soluble complex but never resulted in copolymerization. The copolymer was rather formed with the truncated form of CHMP1B, i.e., in the absence of the MIM1 domain. Therefore the MIM1 domain of CHMP1B is not necessary for the copolymerization with IST1 and may even prevent the formation of the copolymers. However, it is not reported if the MIM1 domain of CHMP1B is free for VPS4 interaction in these copolymers (McCullough et al. 2015). Nevertheless, in the same study the authors confirmed that IST1 active-closed conformation is the one found in either the homo or the hetero-polymers with CHMP1B (Fig. 20B and Fig. 21C, D). This copolymer is a double-stranded tubular filament, with CHMP1B forming the inner sheet and IST1 the outer one of the tube with a highly cationic interior (Fig. 21D). The open CHMP1B conformation is stabilized by inter-subunit interactions along the inner strand. Each CHMP1B protein interacts with four other CHMP1Bs subunits that cross the forearm of the original subunit and the α5 hand interacts with the tip of the hairpin four subunits away, making a domain-swapped contact that is similar to the intra-subunit interaction triggering the auto-inhibition (Fig. 21C). In over-expression conditions, CHMP1B coats positively curved membranes and therefore, the authors speculate about the implication of CHMP1B and IST1 in membrane tubulation from early endosomes (McCullough et al. 2015) (See discussion).

Another interactor of CHMP1B is Calpain-7, a Ca+ dependent cysteine protease. In this case, the α2, but not the MIM1 domain, of CHMP1B interacts with the MIT domain of the Calpain-7. This interaction is further enhanced by the co-expression of IST1 and induce Calpain-7 endosomal localization but nothing is known on the consequences of this interaction, on the structural properties and function of CHMP1B (Yorikawa et al. 2008; Maemoto et al. 2011).

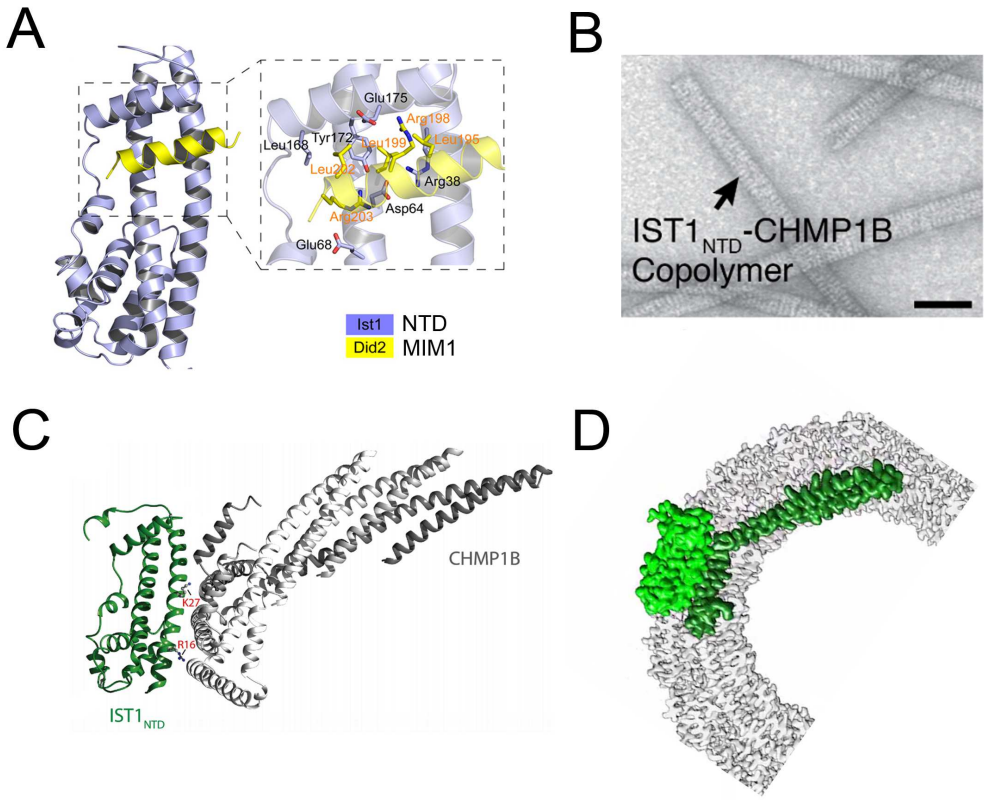


Figure 21: Model for Ist1 and Did2/CHMP1B copolymers: A: The structure of the Ist1NTD/Did2—MIM1 complex (left). Enlarged view of the side chains of the residues at the interface (right). **B:** Negatively stained electron micrographs of wild type CHMP1B –IST1NTD hetero-polymer **C:** Interface between IST1NTD-CHMP1B in the copolymer. IST1NTD residues involved in co-polymer formation are labeled in red. Each Ist1NTD subunit interacts with three different CHMP1B subunits. **D:** End-on view of reconstructed IST1NTD-CHMP1B tube. Single subunits of IST1NTD (light green, outer strand) and CHMP1B (dark green, inner strand) Modified from (McCullough et al. 2015).

3.4.3.4 Examples of Regulation of ESCRT-III Oligomerization by Ubiquitin

In the recent years, post-translational control of oligomerization by the ubiquitin system is rising attention, particularly in a context where the spectrum of proteins which activity is regulated positively or negatively by oligomerization is rapidly increasing and the regulation of the assembly processes *in vivo* is still poorly understood.

There are a few examples of ubiquitin-dependent oligomerization published. No common scheme emerged yet. These studies describe extremely different

mechanisms by which ubiquitin is regulating oligomerization processes exemplifying the huge potential of this post-translational regulation:

(1) Ubiquitination enhances the formation of functional Smad3-Smad4 oligomers and their transcriptional activity *in vivo*. Mono- or oligo-ubiquitination of the K507 of the conserved L3 loop of Smad4 exhibits enhanced ability to oligomerize with R-Smads (Moren et al. 2003). The molecular mechanism of the oligomerization process is not described.
(2) Ubiquitination of the K54 of the DIX domain of Dishevelled blocks its polymerization and activity by interfering with the oligomerization interface *in vitro* (Madrzak et al. 2015)
(3) Mono-Ubiquitination and subsequent ubiquitin binding are two sequential events that induce and stabilize ALIX-V oligomerization *in vitro* and are necessary *in vivo* for its function (see also above) (Keren-Kaplan et al. 2013)
(4) *In vitro* covalent conjugation and non-covalent binding of ubiquitin molecules synergize to induce and stabilize tetramers of immune signaling pathway of a viral RNA sensor tandem caspase activation and recruitment domain Rig-I-(RIG-1-2CARD) (Peisley et al. 2014).
(5) *In vitro* the oligomerization of the Nedd4 ligase depends on the linkage of ubiquitin and causes its inactivation. Indeed, ubiquitin linkage displaces the helix α1 from the oligomerization interface by attraction of its UBD, allowing the formation of a catalytically inactive trimer (Attali et al. 2017).
(6) Different ubiquitination sites have differential effects on the aggregation of α-synuclein, at least *in vitro* (Meier et al. 2012).

3.4.3.5 Assembly of ESCRT-III Filaments

Most of what is known about the ESCRT-III polymer composition comes from yeast studies. The assembly of ESCRT-III and VPS4 at membranes is transient and very flexible, and allows a same set of molecules to induce membranes scission in different biological processes and in diverse manners (Teis et al. 2008; Adell and Teis 2011; Franquelim and Schwille 2017). *In vitro,* most ESCRT-III can form homo- and hetero-oligomers in a concentration dependent manner and preferentially pair at the membrane sites of action as follow: CHMP2-CHMP3, CHMP4-CHMP6 and IST1-CHMP1B (Muziol et al. 2006; Lata et al. 2008a; Lata et al. 2009). During the MVB biogenesis in particular, Vps20 (CHMP6) initiates the polymer, Snf7 (CHMP4) extends it being the major component of the polymer and Vps24 (CHMP3) together with Vps2 (CHMP2) remodel and cap the polymer limiting its length (Teis et al. 2008; Saksena et al. 2009; Henne et al. 2012). In mammalian cells whether the

association of CHMP4 with CHMP2 and CHMP3 can extend the polymer by forming heterotypic or parallel homotypic assemblies is not yet known. CHMP4 can also interact with CHMP5, CHMP1B and IST1 which all play auxiliary roles in the ESCRT pathway and notably contribute to the disassembly of the machinery by interaction with VPS4-LIP5. Moreover, CHMP2, CHMP3, CHMP1 and IST1 are able to individually polymerize *in vitro* forming homo-polymers independently of the presence of CHMP4 (Ghazi-Tabatabai et al. 2008; Lata et al. 2008a; Lata et al. 2008b; Chiaruttini et al. 2015; McCullough et al. 2015). The exact composition of the ESCRT filaments *in vivo* may differs in different situation and/or types of cells.

In vitro, the ESCRT-III filaments in association with GUVs can form flat rings and spirals, helical tubes and conical funnels (Ghazi-Tabatabai et al. 2008; Hanson et al. 2008; Lata et al. 2008b; Teis et al. 2008; Saksena et al. 2009; Wollert et al. 2009a).

Flat rings and Spirals: These structures were notably observed using over-expression of CHMP4 in human cells. CHMP4 forms non-self-limiting filaments of 4.2 nm or mixed 4.9 to 10.6 nm of diameter (Hanson et al. 2008; Henne et al. 2013). The preferred radius of curvature is 21-23 nm and they are stiff enough to function as mechanical springs (Chiaruttini et al. 2015) (Fig. 22).

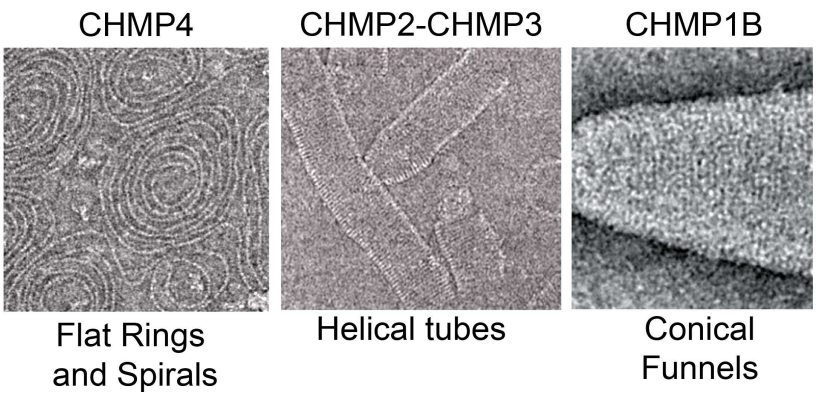


Figure 22: ESCRT-III assembly architectures: *In vitro* the ESCRT-III filaments in association with LUVs or GUVs can form flat rings or spirals, helical tubes and conical funnels. Visualized by EM. Modified from (Schoneberg et al. 2017) and (McCullough et al. 2015).

Helical Tubes were observed in the membrane of human cell over-expressing CHMP4 when the ATPase deficient VPS4 was concomitantly expressed. In these conditions, the diameter of the tube was 100-200 nm (Hanson et al. 2008). In the

case of CHMP2B, its overexpression in human cells induced up to 400 nm tubes (Bodon et al. 2011). Finally, purified CHMP2A and CHMP3 form tubes of around 50 nm *in vitro* (Effantin et al. 2013). The outer surface of these tubes binds the membrane necks and the inner part interacts with VPS4. Helical tubes are also formed by IST1-CHMP1B in complex or by CHMP1B independently of IST1 (as described above). So, the architecture of a given ESCRT polymer may strongly depends on its hetero or homo composition (Bajorek et al. 2009a; Guizetti et al. 2011; McCullough et al. 2015) (Fig. 22).

Conical funnels: In membranes necks, *in vitro* co-assembly of CHMP2 and CHMP3 and CHMP1 homo-polymers form cones of 50 nm diameter on the top and 150 nm on the bottom that bind the membrane with the outer face of the cone (Dobro et al. 2013) (Fig. 22).

ESCRT-III filament are stabilized by both the interaction with the membrane and with other ESCRT subunits in the same filament, including probable lateral interaction of various subunits of different filaments (Ghazi-Tabatabai et al. 2008; Hanson et al. 2008; Lata et al. 2008b; Teis et al. 2008; Henne et al. 2012; Buchkovich et al. 2013; Shen et al. 2014; Chiaruttini et al. 2015; Lee et al. 2015; McCullough et al. 2015; Tang et al. 2015).

To date, however, we still know little about the dynamics of ESCRT-III polymerization: are the filaments composed of hetero-polymers of several core ESCRT-III proteins or do they assemble into parallel homo-polymeric filaments *in vivo?* How these filaments are remodeled during the recruitment to the membrane or during the constriction and the scission of membrane necks? How they are post-translationally regulated? These are a few examples of still open questions that need further investigation to be answered.

3.4.3.6 Membrane Remodeling and Scission by CHMPs

Muziol and colleagues in 2006 noted that the α1 edge of CHMP3 was very cationic (basic) and proposed that this surface binds the membranes. Now, we know that, indeed, ESCRT-III proteins interact with membranes through their basic cluster into their core domain, and some of them, like CHMP4, contain additional N-terminal α0 amphipathic helixes that inset into the regions of positive membrane curvature (Buchkovich et al. 2013; Tang et al. 2015). Most of the ESCRT-III filaments interact with negative curved lipidic membrane necks with the interacting surfaces facing outward of the polymer, except in the case of the filament made by the co-assembly of IST1-CHMP1B that, at least in overexpressing conditions, binds

coat lipid membranes of positive curvature on tubes protruding in the cytosol (McCullough et al. 2015). However, further investigation in more physiological conditions will be necessary to understand the real relation between assembly of ESCRT-III polymers and membrane interaction.

In recent *in vitro* models it has been proposed that Snf7 forms spiral springs that release their energy to deform membranes (Chiaruttini et al. 2015). However, ESCRT-III filaments do not assemble in flat membranes *in vivo*, and therefore, many *in vitro* studies in the absence of ILV neck are physiologically of little relevance even if they can be of big interest to study biophysical properties of the ESCRT-III polymers.

The three current models for membrane scission by the ESCRT-III complexes are:

(1) Dome model: An energetically possible model for membrane scission by the ESCRT-III complexes was proposed by Fabrikant and colleagues in 2009, inspired by the helical-lipid coated tubes formed *in vitro* on GUV by the yeast proteins Vps2 and Vps24 (Fabrikant et al. 2009) and in human cells by over-expression of CHMP4 (Wollert et al. 2009a). In this model an initial deformation of the membrane is necessary for ESCRT recruitments and the basic face of an ESCRT-III dome polymer triggers electrostatic interaction with the lipidic membrane. Due to the interaction with the membrane, the neck of the ESCRT-III tubes could be constricted to a critical threshold of less than 2 nm that would overcome the energetic barrier, and scission would be triggered (Fabrikant et al. 2009). This model however does not explain the presence of VPS4 before scission and the wider and narrower ends of the cone that have been observed in the opposite direction than the one predicted by this model (Cashikar et al. 2014).

(2) Reverse Dome Model: This model works with the same kind of constriction as the dome model but with reverse topology of the dome implying that the ESCRTs are released into the cytosol (Hurley and Hanson 2010; McCullough et al. 2013). The problem here is that the ESCRT-III proteins need to be added in the inner part of the forming ILV and that the growth of the filament will be toward the narrow parts which is energetically unfavorable, so the polymerization should be ATP-dependent which is inconsistent with experimental observations (Chiaruttini et al. 2015; Lee et al. 2015).

(3) Spiral Spring Model: This hypothesis involves mechanical buckling forces applied by the ESCRT-III proteins to the membrane which can directly give rise to membrane invaginations (Carlson et al. 2015; Chiaruttini et al. 2015). Polymer growth would be from narrow to wide. The removal of the first ring by VPS4 would

free CHMP4 to flatten out releasing the energy stored in the membrane and using it to produce the scission (Schoneberg et al. 2017). This model implies that the Vps4 is actively implicated in scission (see next section and Fig. 23).

3.4.3.7 Disassembly of the ESCRT-III Polymers: VPS4

The membrane association of the ESCRT-III is essentially irreversible without the energy procured by VPS4. Indeed, the AAA+ ATPase VPS4 is required for the disassembly of the ESCRT-III polymers in all of the ESCRT-dependent processes. The ATPase mediated disassembly of ESCRT-III is the only thermodynamic input during the membrane deformation and scission processes driven by the ESCRT pathway (Lata et al. 2008b). The dissociation of the ESCRT-I and II from the membrane, in contrast, is not dependent on VPS4 activity (Nickerson et al. 2010).

VPs4 is a dodecameric cylinder with a central pore of conserved amino acid residues required for its function. The enzyme is inactive in monomeric or dimeric state; it oligomerizes and is activated transiently on substrates.

The initial interaction of VPS4 ATPase with ESCRT-III happens through the MIT N-terminal domain of VPS4 and the MIM C-terminal domain of the CHMPs (see Table 2). In humans, the canonical interaction of ESCRT-III with VPS4 occurs through CHMP2B, CHMP1A or IST1 (Obita et al. 2007; Stuchell-Brereton et al. 2007; Schoneberg et al. 2017) and the MIM2 of CHMP6 (Kieffer et al. 2008). *In vitro,* the yeast MIM1 domain of Vps2 (CHMP2) has the highest affinity for Vps4 and is required for disassembly of the complex but not for the scission of the membrane (Teis et al. 2008; Wollert et al. 2009a).

Moreover, in yeast, Did2 functions as an adaptor of Vps4 and is essential for the disassembly of the ESCRT-III lattices but not for the disassembly of ESCRT-I or II (Nickerson et al. 2006).

There are additional proteins that bind Vps4 that are important for its function in membrane remodeling. The better studied is Vta1 (LIP5) that promotes Vps4 oligomerization and includes extra MIT domains (Nickerson et al. 2006; Williams and Urbe 2007; Dimaano et al. 2008; Rue et al. 2008; Xiao et al. 2009).

Strikingly the interaction of Vps4 with Did2 would positively regulate Vps4 while the interaction with Ist1 would have a negative regulatory function on Vps4 recruitment and activity (Dimaano et al. 2008; Agromayor et al. 2009; Bajorek et al.

2009a). Thus, ESCRT-III proteins can either induce or inhibit Vps4 to assembly into active complexes (Azmi et al. 2008; Xiao et al. 2008).

However these observations have been done *in vitro* with isolated proteins and the coordination of the proposed roles for ESCRT and Vps4 remains to be clarified *in vivo*. (See Table 1).

Two main hypothesis explain the function of the Vps4/VPS4 in the disassembly of the ESCRT-III polymer and in the resulting membrane scission:

(1) In the first hypothesis, the disassembly would happen after the scission has been completed and CHMP2 and VPS4 would be needed for repeated rounds of scission (Wollert et al. 2009a; Wollert and Hurley 2010) (Fig. 23A). The depolymerization of ESCRT would happen when VPS4 pulls the ESCRT-III subunits into the pore releasing them from the membrane. The ATP hydrolysis energy would be used to reset the ESCRT-III proteins to the auto-inhibited conformation. This conformation would store energy in the cytosol that will be released as mechanical force upon membrane binding and entry into the filament.

(2) In the second hypothesis, VPS4 acts before and during the scission. Vps4 is necessary for remodeling of the ESCRT filament that will lead to the final scission step. Vps4 could act either as a scaffolding protein or by providing mechanical energy or both (Hanson et al. 2008; Saksena et al. 2009; Nickerson et al. 2010; Adell and Teis 2011).

Currently, is this last model the one that adjust better to the experimental observations. The Vps4 by completely unfolding them and threading them through the central pore (Yang et al. 2015). However, it is not defined whether the Vps4 depolymerization of the ESCRT-III filament is implicated in neck constriction (Fig. 23B model 1: "The Purse String model"). Nevertheless, it has been recently described that the Vps4 depolymerize the ESCRT-III filament by extracting consecutive subunits in a one by one mode (Mierzwa et al. 2017)(Fig. 23B).

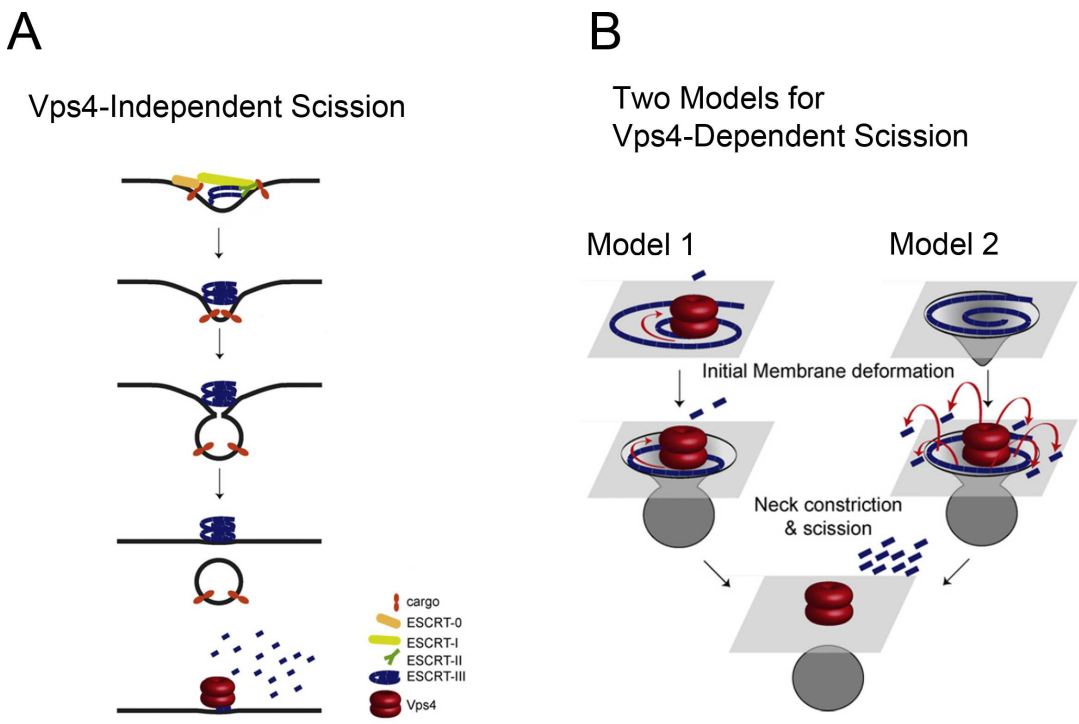


Figure 23: Models for membrane scission by the ESCRT-III complexes: A: The 'Vps4-Independent model": ESCRT-III triggers membrane budding and scission. Vps4 activity is limited to ESCRT-III recycling. **B:** The 'Vps4-Dependent models": Left panel: The 'Purse String model': Vps4 removes one ESCRT-III subunit after another. The shrinking filament constricts and scission the membrane. Right panel: ESCRT-III assembly deforms the membrane and then Vps4 disassembly of the ESCRT-III trigger membrane scission. Modified from (Adell and Teis 2011).

3.5 Post-translational Regulation of the ESCRT Machinery

A number of post-translational modifications of the ESCRT machinery has been reported, such as myristoylation, phosphorylation and ubiquitination that play important roles in their stability, subcellular localization and/or activation.

Main ESCRT members subjected to post-translational regulation are the following:

HRS:

Hrs is both phosphorylated and ubiquitinated. Phosphorylation by the PI3K dependent Pkh1/2 kinases regulates Hrs recruitment at the endosomal membrane (Gasparrini et al. 2012; Meijer et al. 2012). Moreover, the UIM-containing domain of Hrs can be monoubiquitinated in a signaling-dependent manner (Polo et al. 2002). Hrs is phosphorylated by the Syk kinase and ubiquitinated by Cbl. The majority of phosphorylated Hrs is found only in membrane compartments, whereas ubiquitinated Hrs is predominantly cytosolic (Gasparrini et al. 2012). Hrs has also

been reported to be ubiquitinated by AIP4 (Marchese et al. 2003) or POSH causing a reduction of Hrs level via the ubiquitin-proteasome pathway (Kim et al. 2006). Alternatively to proteasome degradation, ubiquitin moieties may mask the UBD of Hrs, inhibiting cargoes recognition and degradation as shown during EGFR trafficking process (Hoeller et al. 2006).

STAM:

STAM undergoes auto-mono-ubiquitination in an E3-independent manner (Hoeller et al. 2007). Since STAM interacts directly with the DUBs AMSH and UBPY these two enzymes may also deubiquitinate STAM (Tanaka et al. 1999; Kato et al. 2000). (See *section 4.5.1 DUBs Implicated in Endocytic Sorting of Receptors*).

TGS101:

TGS101 is mono-ubiquitinated by the E3 ligase Mahogunin both *in vivo* and *in vitro (Kim et al. 2007)*. This ubiquitination plays an important role in the fusion between the lysosomes and the auto-phagosomes or the MVBs (Majumder and Chakrabarti 2015). However, the molecular mechanism of this regulation is not described. Other studies show that ubiquitination of Tsg101 could be triggered also by the E3 ligase Tal (McDonald and Martin-Serrano 2008) and that ubiquitin linkage would induce its dissociation from endosomes inhibiting its function (Amit et al. 2004). Interestingly, the herpes simplex virus ubiquitin protease VP1/2 hydrolyses ubiquitin moieties on TSG101 inducing its nuclear localization and activation illustrating the co-evolution of the host and a pathogen (Calistri et al. 2015).

Alix:

ALIX-V dimerization and multimerization are needed for its activity in HIV budding and TSG101 binding and depend on its ubiquitination (Carlton and Martin-Serrano 2007; Pires et al. 2009). Indeed, ALIX-V, which contains an UBD, undergoes an E3-independent ubiquitination; then, the association of ubiquitin to the UBD of Alix induces and stabilizes its oligomerization. The two ALIX-V-binding patches on the ubiquitin surface provide a molecular explanation for the stabilization of ALIX-V in oligomers.

CHMP6:

CHMP6 is myriostilated *in vivo* in its N-terminal half which is crucial for its recruitment to the endosomal membranes through the interaction with CHMP4B and EAP20 (Yorikawa et al. 2005).

CHMP1A/B, CHMP2A/CHMP4C and Ist1 phosphorylation:

The kinase ULK3 binds MIM domains of CHMP1A, CHMP1B, CHMP2A, CHMP4C and IST1 and phosphorylates the corresponding proteins. CHMP4C is also a substrate of the Aurora B kinase. In the case of Ist1 and CHMP4B, this phosphorylation is necessary for cytokinesis regulation. Specifically, ULK3 phosphorylation of IST1 delays cell abscission by regulating the interaction of IST1 with the VPS4-LIP5 complex. The function of CHMP1B phosphorylation has not yet been described, however CHMP1B interaction with ULK3 is necessary for the function of ULK3 in cytokinesis (Caballe et al. 2015). Finally, in both human cells and *Drosophila*, the Aurora-B kinase phosphorylates CHMP4c/Shrub and regulate cell abscission (Carlton et al. 2012; Matias et al. 2015).

The assembly of ESCRT-III in mammalian systems may be more complicated than the model proposed from studies in the yeast system due to the presence of additional ESCRT-III components: CHMP7 and CHMP5, two isoforms in CHMP1 and CHMP2 and three isoforms in CHMP4. And therefore, most probably the post-translational regulation of these subunits are key pieces in the regulation of the assembly of ESCRT-III. Of note, no ubiquitination of ESCRT-III has been reported previously to this work.

3.6 Methodological Problems Encountered for the *in vivo* Study of the Mammalian ESCRT Machinery.

I find important to mention some methodological problems in the study of the ESCRT machinery. All our knowledge come mainly from studies using yeast model or purified yeast proteins and *in vitro* assays.

For the study of the ESCRT function in mammalian cells, RNA interference has been extensively used but human cultured cells usually survive knockdown of even multiple silencing of core ESCRT genes (Morita et al. 2011). Given the variability in the efficiency of the knockdowns of proteins, the absence of a phenotype is however not necessarily a robust evidence for the ESCRT independence of a given pathway.

Moreover, given the wide spectrum of cellular functions that a unique ESCRT protein can carry out, the experiments do not differentiate between direct contribution to the process under study and the scope of the indirect contributions to the observed phenotypes caused by the perturbations of other cellular functions.

In the particular case of the CHMPs, overexpression of GFP-tagged ESCRT-III subunits has been shown to be inhibitory as a result of dominant-negative function of the corresponding recombinant proteins. Therefore, this is typically considered something to be avoided in imaging studies, but actively pursued for functional inhibition (Martin-Serrano et al. 2003; Strack et al. 2003; Zamborlini et al. 2006). GFP is a big protein, but small tags, like HA or Myc, also interfere with the function of CHMPs *in vivo*. For instance, overexpression of a Myc or HA tagged-CHMP1 in *Drosophila* is not able to rescue the developmental phenotype induced by the *Chmp1* gene knockdown, while over-expression of non-tagged protein does (Valentine et al. 2014). Consequently, with poorly efficient antibodies, the detection of expressed or endogenous CHMPs *in vivo* remains extremely difficult. For these reasons, most of the analysis performed so far belong to biochemical scope, addressing what components are minimally sufficient to trigger membrane remodeling or other ESCRT function, rather than what is essential in the context of a minimal systems. Spatial or temporally restriction of the systems and transgenic expression of essential ESCRT genes in living organisms such as *Drosophila* are slowly pushing the functional study of the CHMPs forward (Matusek et al. 2014; Loncle et al. 2015; Matias et al. 2015).

4. UBIQUITIN SYSTEM IN ENDOCYTOSIS OF MEMBRANE RTKs: THE EGFR CASE

4.1 Introduction

Receptor tyrosine kinases (RTKs) are high-affinity cell surface receptors for many growth factors, cytokines and hormones. Approximately 20 different RTK classes have been identified. The main ones are:

- RTK class I (EGF receptor family) (ErbB family)
- RTK class II (Insulin receptor family)
- RTK class III (PDGF receptor family)
- RTK class IV (VEGF receptors family)
- RTK class V (FGF receptor family)
- RTK class VI (HGF receptor family)
- RTK class VII (Trk receptor family)
- RTK class VIII (Eph receptor family)

The most comprehensive studies of RTKs endocytosis have been done using the EGF/EGFR pathway as an experimental model in human cells.

The EGFR (or ErbB1) in particular is a member of the ErbB growth factor RTK class I involved in multiple downstream signaling events controlling various cellular processes such as mitogenesis, cell survival, differentiation and cell migration. These processes must be tightly regulated during both development and adult life; their deregulation notably favor tumorigenesis and cancerogenesis. The main mechanism by which cells downregulate activated receptors is by ligand-mediated endocytosis and the subsequent delivery of the complex to the lysosome for degradation (Alwan et al. 2003; Jorissen et al. 2003).

Since the cloning of the EGFR and the first analysis of its endocytosis (Carpenter and Cohen 1976b; Carpenter and Cohen 1976a), a growing number of studies have been published on the mechanisms of EGFR endocytosis and the role of the endocytosis in the regulation of EGFR signaling.

The role of EGFR ubiquitination during the internalization is controversial due to co-existence of ubiquitin-dependent and independent internalization signals (Duan et al. 2003) (See also below). However, it is widely accepted a model whereby cycles of ubiquitination and deubiquitination of the EGFR and of the endocytic

machinery itself regulate EGFR trafficking towards the lysosomes, and therefore, the duration of downstream signaling. For review see (Madshus and Stang 2009; Ceresa and Peterson 2014; Bergeron et al. 2016). The ubiquitination status of the EGFR is the result of a balance between the activity of the E3 ligases and the deubiquitinases implicated in its regulation (Komada 2008).

4.2 Dynamic Maintenance of the Basal Levels of (non-activated) EGFR in the Plasma Membrane

The presence of EGFR at the cell surface is mostly determined by the rate of three trafficking processes: the delivery to the PM of newly synthetized EGFR, the internalization of EGFR from the PM and its recycling to the PM. In the resting state, the EGFR undergoes constitutive internalization from the PM at rate similar to other integral PM proteins driven by basal membrane recycling (internalization rate constant ke of about 0.02 to 0.05 min^{-1}). This rate is however slower than the constitutive recycling rates back to the PM, so the EGFR would accumulate in the PM increasing its accessibility to its ligands over time, as observed at least in cultured cell models (Herbst et al. 1994; Goh and Sorkin 2013). In human cultured fibroblasts for example, in the resting state, most EGFRs reside on the cell surface with a fraction of the receptors (2-3% each minute) being slowly internalized by endocytosis and quickly recycled back to the PM ($t_{1/2}$ of approximately 5 min). However, the half-life of the RTKs depends both on the cell type and the nature of the RTK considered. In fibroblasts or Keratin-forming tumor cell line HeLa (KB) cultured cells, moderately overexpressed EGFR (<200,000 units per cell) exhibits a turn-over with a $t_{1/2}$ of 6-10 h. In contrast, carcinoma A-431 cells overexpressing EGFR display a turn-over with a $t_{1/2}$ of about 24h, illustrating the ability of some cancerous cells to stabilize the EGFRs (Stoscheck and Carpenter 1984a; Stoscheck and Carpenter 1984b).

Activated EGFR, i.e., in its phosphorylated form (pEGFR), in contrast to the non-activated EGFR, shows a slow rate of recycling ($t_{1/2}$ of about 10-23 minutes instead of 5 min) due to its retention within recycling endosomes. Endosomal retention of EGFR correlates with lysosomal targeting of its ligand EGF, and therefore to the termination of downstream signaling (Herbst et al. 1994; Wiley 2003).

4.3 Endocytosis of Activated EGFR

4.3.1 EGFR Activation and Downstream Pathways

EGFR, ErbB3 and ErbB4 are activated by ligand-induced homo- or hetero-dimerization, while in the case of ErbB2, this receptor is activated through hetero-dimerization with another ligand-bound ErbBs (Yarden and Sliwkowski 2001). The ligand-induced endocytic trafficking of activated EGFR is the best characterized among ErbBs.

At least six families of EGFRs ligands have been reported: The EGF, Amphiregulin, the Transforming growth factor alpha (TGF-α), Betacellulin, the Heparin binding EGF-like growth factor (HB-EGF), and Epiregulin (Sweeney and Carraway 2000). Depending on the signaling molecule and the type of EGFR dimers different pattern of phosphorylated EGFR will be achieved (Olayioye et al. 1998). This determines the downstream signaling pathway(s) that will be activated because the 5 to 8 amino acids surrounding the phosphorylated tyrosine reside (pY) confer the specificity of Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains binding (Songyang et al. 1993). In the following section, I will focus on EGF-mediated activation of EGFR.

The EGFR is a type I receptor with an extracellular N-terminus and an intracellular C-terminus. Ligand binding to the extracellular domain induces a conformational change that promotes receptor dimerization and subsequent activation of the receptor's intrinsic tyrosine kinase domain. Then, the kinase domain of one receptor phosphorylates the tyrosine residues on the carboxyl terminus of its partner at multiple sites such as Y1045, Y1068 or Y1086 besides others (Fig. 24).

Phosphorylated EGFR (pEGFR) recruits a number of proteins containing SH2 and PTB domain which binds to the pY residues. These proteins consist in both adaptor proteins, such as SHC transforming protein 1 (Kuz'mina et al.), Growth factor receptor-bound protein 2 (GRB2), Docking protein 2 (DOK2) and NCK adaptor protein 1 (NCK1), and enzymes, such as c-Cbl, Phospholipase C gamma 1 (PLC-gamma 1), v-Src sarcoma viral oncogene homologous (c-Src) and PTK2 protein tyrosine kinase 2 or the phosphatidyl inositol 3-kinase (PI3K) (Olayioye et al. 2000; Yarden and Sliwkowski 2001; Marmor et al. 2004) and other trafficking signals. The pY also serve as docking sites for other downstream signaling molecules like the enzymes phosphatidylinositol 3-kinase (PI3K), phospholipase C gamma (PLCg), Src, and c-Cbl (Olayioye et al. 2000; Yarden and Sliwkowski 2001) and other trafficking signals (Fig. 25). The three best characterized signaling pathways induced through EGFR activation are the Ras–mitogen-activated protein

kinase (Ras-MAPK), the phosphatidylinositol 3' kinase-protein kinase B (PI3K-PKB/Akt), and the phospholipase C–protein kinase C (PLC-PKC) pathways. A simplified scheme of EGFR downstream signals can be seen in Figure 25. Reviewed in (Kandasamy et al. 2010; Lemmon and Schlessinger 2010) and are drown in details here: **http://www.netpath.org/netslim/EGFR1_pathway.html**

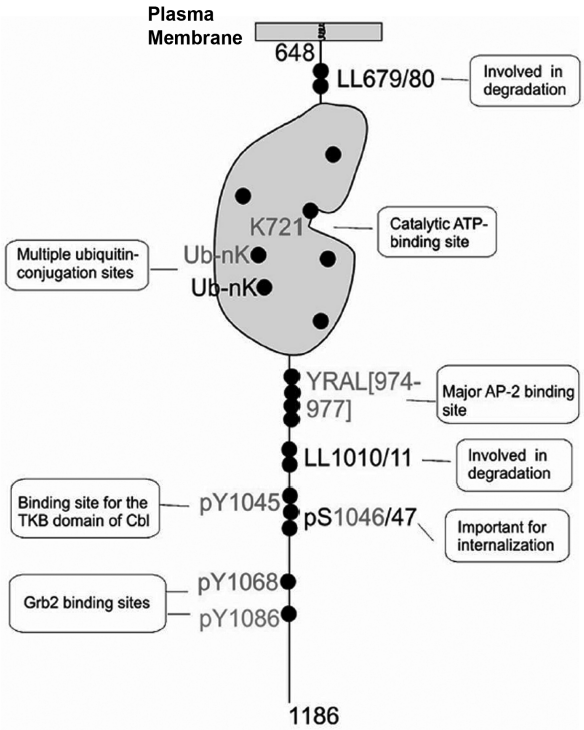


Figure 24: Intracellular motifs of the EGFR (residues 648–1186). Modified from (Sorkin and Goh 2008).

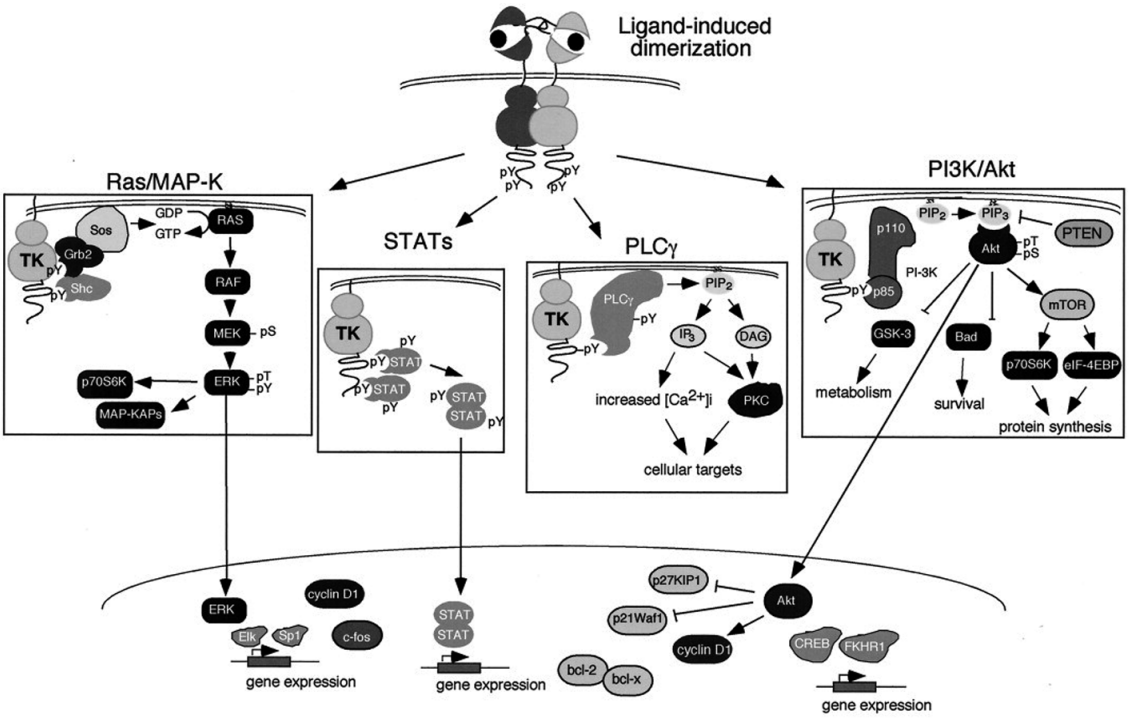


Figure 25: EGF-EGFR induced signaling pathways. Upon activation of tyrosine kinase activity and the phosphorylation of specific tyrosine residues (pY) within the carboxyl-terminal tail of EGFR, signaling effectors containing binding pockets for pY-containing peptides (either SH2- or PTB- domain proteins) are recruited to activated receptors and induce the various signaling pathways activated: Ras-MAPK, PI3K-Akt, PLC-PKC, and the STAT pathways. (Marmor et al. 2004).

4.3.2 Mechanisms of EGFR Ubiquitination

By tandem mass spectrometry (LC-MS/MS), the laboratory of Alexander Sorkin has identified six lysine residues (K692, K713, K730, K843, K905, and K946) in the surface of the kinase domain of the EGFR that can be ubiquitinated at the level of the PM in response to 2 min of EGF stimulation (Fig. 24) (Huang et al. 2006). EGFR ubiquitination starts at the plasma membrane and continues at the level of the endosomes (Umebayashi et al. 2008; Madshus and Stang 2009).

This is the ligand-induced dimerization and transphosphorylation of EGFR in its C-terminal intracellular tail which causes the association of the RING domain E3 ubiquitin ligases Cbl and the subsequent linkage of ubiquitin to the EGFR (Levkowitz et al. 1998). The binding of Cbl to the pY1045 of the EGFR occurs

through the SH2 or the PTB (phospho-tyrosine binding) domain of Cbl (Thien and Langdon 2001). Alternatively, Cbl can be indirectly recruited through the adaptor protein Grb2 (Fig. 24, 26 and 27)(Huang and Sorkin 2005; Huang et al. 2006). Grb2 SH3 domain is actually responsible for coupling the membrane domains containing EGFR with downstream effectors involved in internalization process (Yamazaki et al. 2002).

Some studies show that direct recruitment of Cbl to pY1045 leads to EGFR monoubiquitination on multiple lysine residues (multi-mono-ubiquitination) (Haglund et al. 2003b; Mosesson et al. 2003; Huang and Sorkin 2005) while others show that the recruitment of Cbl occurs through Grb2 which is indispensable for the ubiquitination of the EGFR (Sigismund et al. 2013). Another study shows that a fraction of the EGFR would be modified with K63-linked poly-ubiquitin chains within the kinase domain (Huang et al. 2006).

In the following paragraph, I will use the case of the EGFR and its ligand EGF to describe how endocytosis and the ubiquitin system are implicated in the interpretation of EGF signaling concentrations in human cell cultures.

4.3.3 Threshold-Controlled Ubiquitination of EGFR Determines EGFR Fate

The biological outputs that a cell can present in response to different concentration of a given signal depends on the biological context. Some signals have to be interpreted just as momentum information and others as both time and place information.

To face these requirements, responses to extracellular signaling molecules can be processed in two manners by the cells: (1) The cellular response is always the same but proportional to the concentration of the signaling molecule or (2) the cell transforms gradients of the signaling molecule into a switch-like threshold-activated responses allowing for different kinds of response.

An example of the situation (1) is the phosphorylation of the EGFR in response to EGF. EGFR Phosphorylation follows a sigmoidal phosphorylation response to signal.

An example of the situation (2) is the ubiquitination of the EGFR at the PM in response to EGF. Indeed, the phosphorylation of two tyrosine residues on the same EGFR is necessary for its ubiquitination.

Therefore, the ubiquitin system acts as a second step sensor mechanism which will transform the sigmoidal response into a switch-like response: if ubiquitination is triggered, clathrin independent endocytosis of the receptor will be triggered. In contrast, clathrin mediated endocytosis of EGFR is independent of ubiquitination and is triggered independently of the EGF concentration (Sigismund et al. 2008; Sigismund et al. 2013).

4.3.4 Role of the Ubiquitination of EGFR at the Plasma Membrane: CME or CIE?

Low concentration of EGF (from 1 to 20ng/ml) result in the rapid clustering of the phosphorylated EGFR complexes in the clathrin-coated pits followed by their internalization trough CME (Miller et al. 1986). The internalized EGFR is then mainly sent back to the membrane and keeps computing the extracellular signal. The CME of EGFR is therefore necessary for sustained EGFR signaling (Sigismund et al. 2008; Sigismund et al. 2013).

At higher concentrations of EGF (for instance 100 ng/ml) or in EGFR over-expressed conditions, both CME and CIE of the activated EGFR complexes are observed (Sigismund et al. 2005; Sigismund et al. 2008). CME and CIE deliver receptors to early endosomes located at the cell periphery preferentially (Hopkins et al. 1985). From there, the EGFR can be send back to plasma membrane through the fast recycling compartment as notably the case in CME. Alternatively, the receptors can be redistributed to perinuclear endosomes and MVB promoting their delivery to the lysosomes and degradation, as notably the case in a CIE internalization process (Miller et al. 1986; Carter and Sorkin 1998) (Sorkin et al. 1991).

Thus, CIE induces EGFR degradation and protects the cells from overstimulation by committing most of the receptors for lysosomal degradation (Sigismund et al. 2008; Sigismund et al. 2013).

Which endocytic pathways is activated depends on EGFR ubiquitination which itself depends on the EGF concentration. Indeed, the ubiquitination of the EGFR at the PM is only detectable at high dose of EGF and is the tag responsible for inducing the CIE of the EGFR and its subsequent degradation (Haglund et al. 2003a; Mosesson et al. 2003; Huang and Sorkin 2005; Sigismund et al. 2005; Sigismund et al. 2008) (Fig. 26).

Sensing EGF concentration: triggering or not ubiquitination of the receptor?

EGFR phosphorylation follows a sigmoidal phosphorylation response to signal (Sigismund et al. 2008; Sigismund et al. 2013).

The generation of a sigmoidal response depends on the phosphorylation of one to three tyrosine residues of the EGFR. If the phosphorylation of each individual Y follows a linear dose-response curve, and each phosphorylation event is independent of the others, the probability of having two pY in the same EGFR increases non-linearly as a function of EGF concentration. Since the presence of at least two pY is needed for the activation and recruitment of the Cbl E3 ligase to EGFR, the cell couples the exponential phase of EGFR phosphorylation during EGF stimulation to the ubiquitination event. The mechanistic of this coupling works thanks to the specific association of Grb2 to pY1068 or pY1086 and of Cbl to pY1045. The complex Cbl:Grb2 will produce ubiquitination of the receptor as soon as any combination of pY1045 with pY1086 or pY1068 exist. Binding of Cbl to the pY1045 can happen independently of the binding of Grb2 but is insufficient to induce the ubiquitination of the EGFR. Indeed, activation of the catalytic activity of Cbl is Grb2 association dependent. When ubiquitination is achieved, the CIE of the EGFR is triggered (Fig. 26).

In the case of the CME of the EGFR, Cbl/Grb2 association to EGFR is necessary but it does not trigger ubiquitination of the EGFR (Huang and Sorkin 2005). So, in this specific case, the ubiquitination of some other proteins than EGFR by the Cbl/Grb2 complex may trigger CME (Dikic and Schmidt 2007; Huang et al. 2007; Sorkin and von Zastrow 2009) (Jiang and Sorkin 2003). Few data have been published however about this matter. In one study, Savio and collaborators show that Esp15 ubiquitination status, which is controlled by the dual activity of the Nedd4 ligase and USPX9 deubiquitinating enzyme, plays an important role in the EGFR internalization by CME (Savio et al. 2016). How EGF signaling triggers Nedd4 recruitment and activation is not described. But in another study, Duan and collaborators showed that ubiquitination of Eps15 is not necessary for the CME of the EGFR and that this process is fully independent from the association of Cbl to the EGFR (Duan et al. 2003). Finally, other studies have shown that the Cbl RING domain acts as an adaptor to recruit other important proteins like endophilins to the PM and trigger CME of the EGFR (Soubeyran et al. 2002; Huang and Sorkin 2005) (Fig. 27).

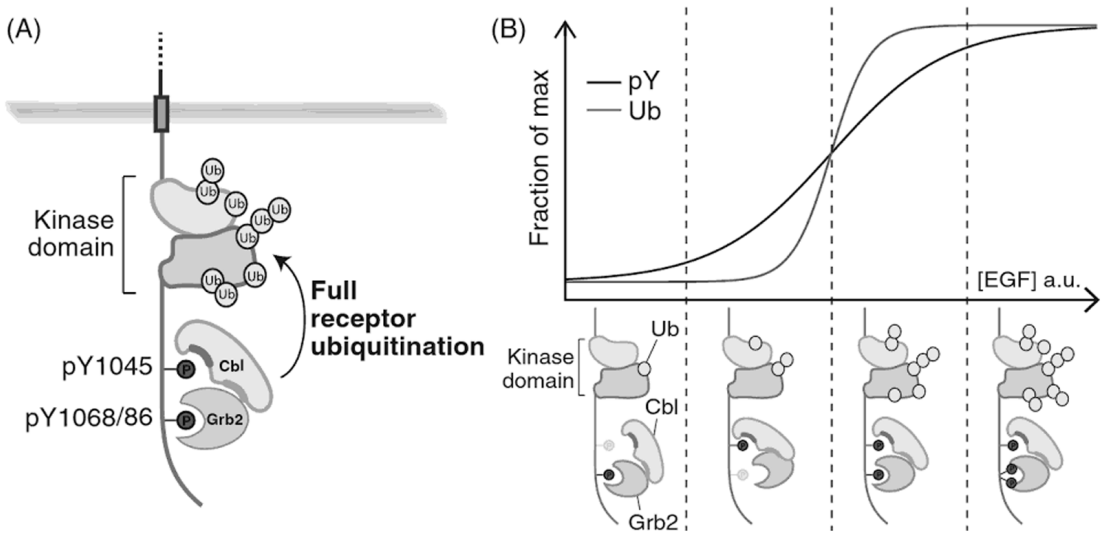


Figure 26: Plasma Membrane EGFR ubiquitination in response to EGF: A: Schematic representation of the cooperative binding model between Cbl/Crb2 complex and the EGFR that would allow full ubiquitination of the EGFR. **B:** A probabilistic model shows how the EGFR ubiquitination threshold is generated. (Modified from A Conte and S Sigismund Ubiquitination and transmembrane signalling Book July 2016)

More recent studies have proved that actually CME of the EGFR is regulated by four redundant and cooperative mechanisms: (1) ubiquitination of the receptor kinase domain, (2) the clathrin adaptor complex AP-2, (3) the Grb2 adaptor protein for Cbl, (4) the acetylation of 4 C-terminal lysine residues (Goh et al. 2010).

Moreover, some studies show that a single ubiquitin molecule is enough to trigger the EGFR internalization (Haglund et al. 2003a; Haglund et al. 2003b; Mosesson et al. 2003). Other studies show that from the total EGFR ubiquitinated upon EGF stimulation, more than 50% were in a form of polyubiquitin chains, mostly K63 and also some K29 chains (Huang et al. 2006; Chastagner et al. 2008; Lauwers et al. 2009).

A simplified scheme of the minimal features characteristics of every ubiquitin-dependent EGFR internalization routes is represented in Figure 27.

What is the mechanism by which ubiquitination of the EGFR subsequently targets triggers CIE rather than CME is not fully clarified. It seems that saturation of the CME plays a role in sending the activated EGFR to the CIE pathway when the threshold of EGF concentration has been reached (Schmidt-Glenewinkel et al. 2008).

Moreover, proteins containing UBD like Eps15/Eps15R and Epsine1 which bind both to ubiquitinated EGFR (Ub-EGFR) and to clathrin and AP2 contribute to the early endocytic step in CME (Fig. 28) (Sigismund et al. 2005; Huang et al. 2006; Madshus and Stang 2009).

Epsin-1 in particular is involved in the translocation of Ub-EGFR from the PM to clathrin-coated areas, and thereby, promotes endocytosis (Kazazic et al. 2009) et al., 2009)(Hawryluk et al. 2006). Packaging of the receptors and membrane deformation and/or microdomains may also have a role in the early internalization of EGFR (Orth et al. 2006; Kerr and Teasdale 2009)(Fig. 28).

Presently, although the role of Grb2 and E3 ligase Cbl in EGFR endocytosis is well established, the understanding of the manner by which ubiquitination directs the early internalization step needs further mechanistic studies.

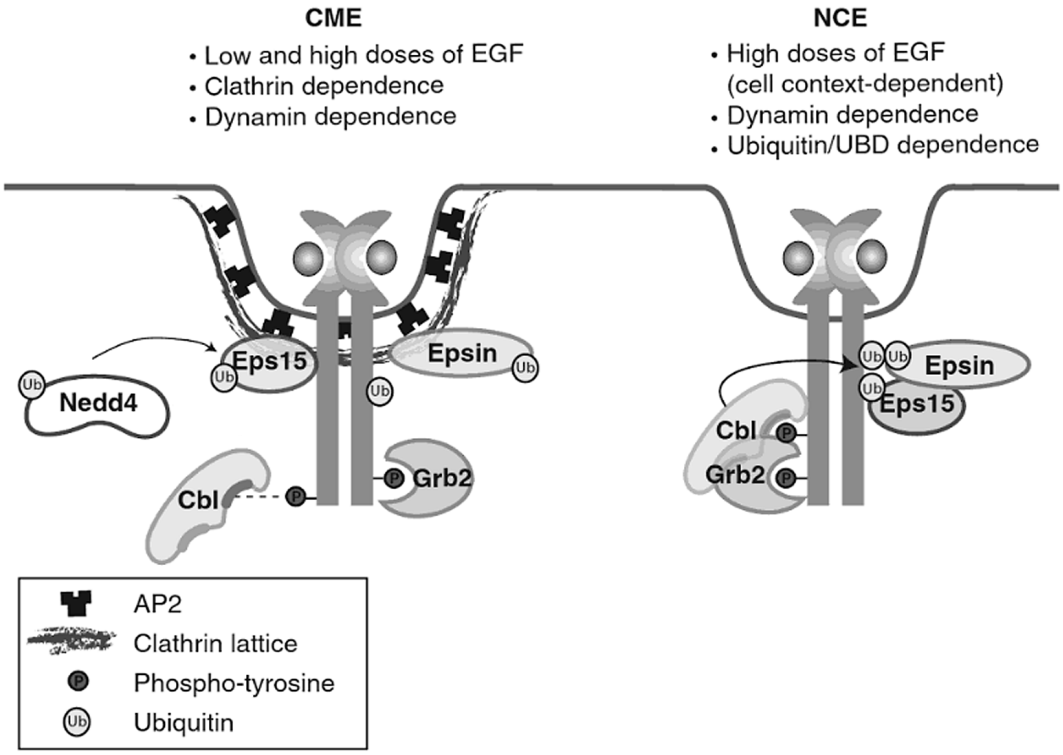


Figure 27: Ubiquitin-dependent EGFR internalization routes: Left: Canonical CME pathway is activated at any given dose of EGF. Eps15 and Epsins interact with AP2 and regulate the formation of CCPs. **Right:** CIE pathway is activated only at high doses of EGF. There is a massive receptor phosphorylation and ubiquitination and a stable association of the Cbl/Grb2 complex. Eps15 and Epsins are responsible for the recognition of the ubiquitinated receptor through their UBD and drive its internalization. (Modified from A Conte and S Sigismund Ubiquitination and transmembrane signalling Book, July 2016)

4.3.5 Role of Ubiquitination in EGFR Sorting: Degradation or Recycling?

Although it exists some controversy about how the ubiquitination of the EGFR directs its internalization, and if other kinds of ubiquitination than monoUb exists, it is well established that the sorting ratios of EGFR are ubiquitin-dependent.

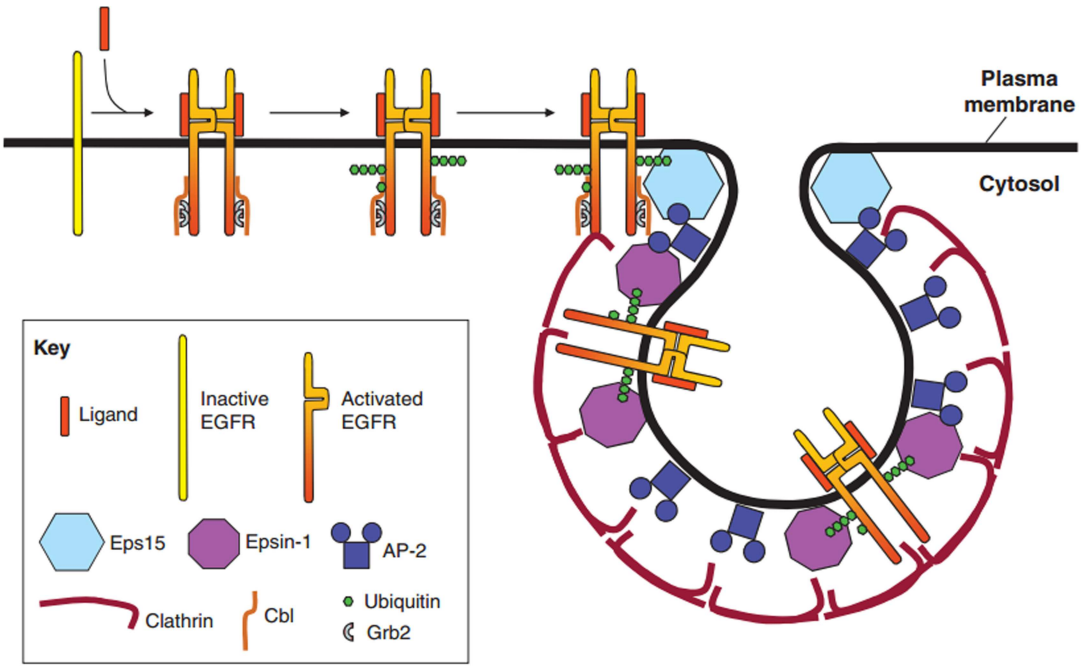


Figure 28: Recruitment of EGFR into CCPs. In the model presented, ligand binding to EGFR induces receptor dimerization and kinase activity. Kinase activity results in the phosphorylation of tyrosine residues in the EGFR tail, allowing Cbl to bind directly, or indirectly through Grb2. Recruitment of Cbl results in mono- and polyubiquitination of the EGFR tail. PolyUb chains can interact with UBD of epsin-1 and Eps15. Because Eps15 is localized at the rim of clathrin-coated pits ubiquitinated EGFR initially interacts with Eps15. Then EGFR is transferred to epsin-1 and thereby recruited into the central region of CCPs (Kazazic et al. 2009). The clathrin-coated membrane subsequently invaginates and is released into the intracellular media as a CCV (Madshus and Stang 2009).

In addition, EGFR sorting can be signal-dependent. For example the PKC-dependent phosphorylation of Y-654 drives the EGF-EGFR complexes from

degradation to recycling (Bao et al. 2000); the association of EGFR with α5β integrin via RCP can also induce its recycling (Caswell et al. 2008).

A. Degradation:

Lysosomal sorting of receptors is a crucial event negatively regulating EGFR signaling because it segregates the kinase from its cytosolic substrates and induces the degradation of EGFR(Madshus and Stang 2009). Ubiquitination is the main player of EGFR degradative sorting and this is directly a consequence of the nature and mechanisms of action of the sorting machinery that contain UBDs recognizing the ubiquitinated EGFR and/or ubiquitinated endosomal adaptor proteins. At the level of the MVBs in particular, the sorting machinery is composed of the four ESCRT complexes and three of them contain Ub-binding subunits (Hicke and Dunn 2003; Stuffers et al. 2009).

In porcine aortic endothelial (Shim et al.) cells overexpressing the human EGFR, the average half-life of the EGFR upon EGF stimulation at high doses is around 2h. By contrast, the degradation of a Ub-defective EGFR (where all the K are mutated to R) is delayed to 4h. Since the difference in the EGFR degradation kinetic is not due to defects in the internalization process, the authors conclude that EGFR sorting to the lysosomal pathway must be altered in the absence of ubiquitin linkage to the EGFR (Huang et al. 2006). Indeed, these authors show that after 10 min of EGF stimulation both the wild type and the Ub-defective EGFR are located in the early endosomes. However, after 3h of EGF stimulation, 70% of the wild type receptor is found in the late endosome, while the Ub-defective mutant mostly stays in the early endosomes compartment (Huang et al. 2006; Eden et al. 2012). The authors propose that EGFR accumulation in the early endosomal compartment leads to an increased recycling back to the plasma membrane (Huang et al. 2006).

Importantly, EGFR seems to be continuously deubiquitinated and reubiquitinated *en route* to the lysosome (Longva et al. 2002). After its internalization, when interaction with the ESCRT-0 Hrs occurs, EGFR is partially deubiquitinated and the ligand-bound EGFR is specifically reubiquitinated probably by UBE4B at the endosomal membranes (Longva et al. 2002; Madshus and Stang 2009; Sirisaengtaksin et al. 2014)(Fig. 29).

Actually, the ultimate formation of the EGFR containing ILVs into the MVBs is a process which depends on the deubiquitination of the receptor, and this is also true in the case of the slow recycling pathway that starts out from the membrane of

the late endosomes. Reviewed in (Madshus and Stang 2009) and (Goh and Sorkin 2013). (See *section 4.3 for a detailed description of the role of DUBs in EGFR sorting*).

B. Recycling:

Due to huge technical difficulties in recycling rates measurements, the field is not strongly documented. Nevertheless, the kinetics of EGFR recycling that have been reported are in line with the theory that late endosomal recycling is the default pathway for EGFRs that are not trapped by the ESCRT-0 in the MVBs (Huang et al. 2006; Goh and Sorkin 2013).

This hypothesis has been setup from the observation that unoccupied receptors are more recycled than the occupied ones (Resat et al. 2003). The EGF-EGFR complexes can however be recycled back to the PM from early/sorting endosomes thanks to the constitutive formation of recycling vesicles from this compartment. The EGF-EGFR complex can also be recycled with slower kinetics from the perinuclear late endosomes/MVBs in which case, it involves a sorting step at the MVB (Sorkin et al. 1991). The ubiquitin tag determines the recycling of the complexes: in early endosomes, non-ubiquitinated EGF-EGFR complexes are recycled to the plasma membrane or directed to other intracellular compartments while ubiquitinated EGF-EGFR complexes are sorted to the late endosomes (Hicke and Dunn 2003; Piper and Luzio 2007).

Since the lysosomal degradation pathway can saturate, the recycling pool of EGF-EGFR complexes may also be proportional to its intracellular amounts or, as mentioned above in the case of the Ub-defective EGFR mutant, to defective sorting to lysosomal pathway (Eden et al. 2009; Eden et al. 2012).

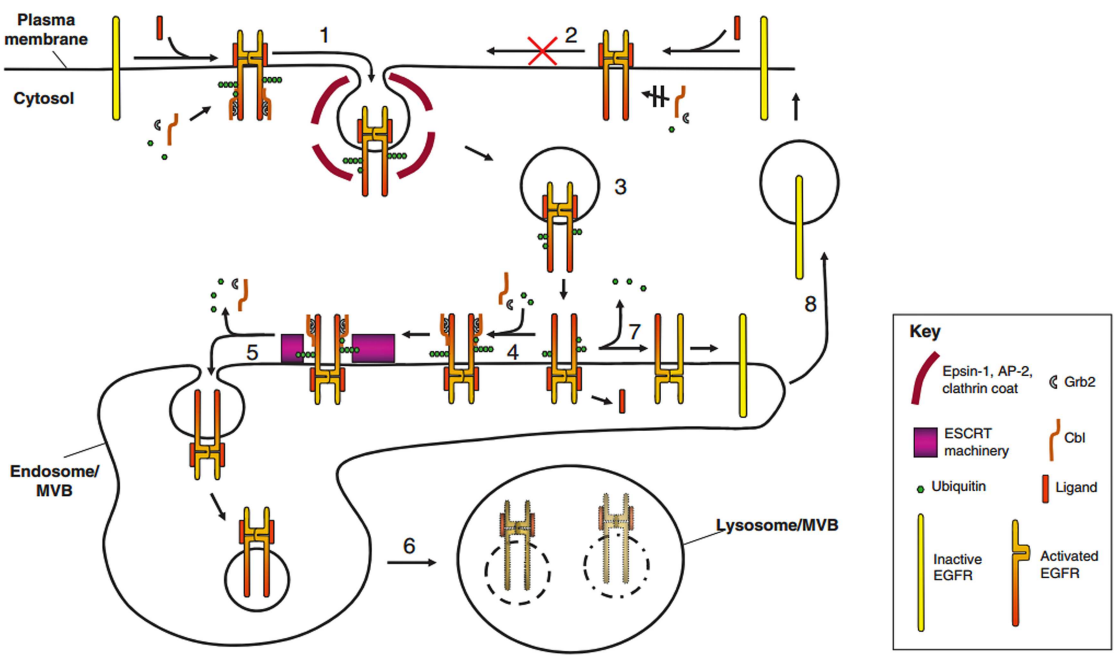


Figure 29: Ubiquitination-Deubiquitination events implicated in endosomal trafficking of EGFR. Upon EGF binding, EGFR is activated, ubiquitinated and recruited into CCPs **(1)**. However, when ubiquitination of activated EGFR is blocked, its recruitment into coated pits is inhibited **(2)**. EGFR is partially deubiquitinated after internalization **(3),** but that only ligand-bound EGFR is reubiquitinated probably by UBE4B (Longva et al. 2002; Sirisaengtaksin et al. 2014) **(4).** Interaction of EGFR with ESCRT machinery on endosomes leads to sorting into ILVs at the MVBs **(5)** and finally to lysosomal degradation **(6).** If the EGFR ligand dissociates EGFR that localizes to endosomes is deactivated and deubiquitinated **(7)** and recycled **(8)** to the plasma membrane (Pickart and Eddins 2004; Madshus and Stang 2009).

4.4 The role of DUBs During Receptors Trafficking

Ubiquitination is the main entry signal for the receptors into the endocytic pathway and consequently, regulation of the ubiquitination status of the cargoes plays also an important regulatory role. This is actually also true for the endocytic machinery itself: DUBs can hydrolyze and/or remodel the ubiquitin chains on members of the ESCRT machinery as at least shown for Hrs (McCullough et al. 2006; Urbe et al. 2006; Zhang et al. 2014).

Two main stages of the receptor endocytosis are regulated by DUBs: (1) the different kinds of internalization and (2) the differential sorting at the late endosome/MVB membranes.

4.4.1 DUBs Implicated in Endocytic Sorting of Receptors

Currently five DUBs have been directly or indirectly implicated in the EGFR endocytosis: AMSH and UBPY, Cezanne, USP2, and USP9x. In addition, USP18 is important for the regulation of the expression of the EGFR.

The JAMM family member AMSH (associated molecule with the SH3 domain of STAM) and the ubiquitin-specific protease (USP) family member USP8/UBPY are the main DUBs implicated in the regulation of EGFR ubiquitination and degradation.

Both AMSH and UBPY contain N-terminal MIT domains and interact with the ESCRT-0 Hrs-STAM complex *via* the same SH3 domain of STAM. They are also recruited by Bro1/Alix and different ESCRT-III members and might each have dual effects on EGFR degradation (Amerik et al. 2000; Kato et al. 2000; McCullough et al. 2004; McCullough et al. 2006; Mizuno et al. 2006; Row et al. 2006).

4.4.1.1 AMSH

AMSH (associated molecule with the SH3 domain of STAM) is a member of the JAMM family of DUBs which specifically cleaves K63 and K48 ubiquitin chains (McCullough et al. 2004; McCullough et al. 2006).

AMSH interacts with the SH3 domain of its substrate, the ESCRT-0: STAM. This interaction needs the UBD of STAM and activates AMSH catalytic activity *in vitro*. Deubiquitination of STAM stabilizes the protein complex Hrs-STAM and would thus promote EGFR degradation in living cells (McCullough et al. 2006; Row et al. 2006; Row et al. 2007). (Davies et al. 2013). AMSH may also deubiquitinates the EGFR itself, thus, in this case rather playing opposite function by preventing EGFR sorting to MVB and its subsequent degradation (Mizuno et al. 2005; McCullough et al. 2006).

AMSH also interacts with the ESCRT-III members CHMP1A and CHMP1B with low micro-molar affinities (Solomons et al. 2011), and with CHMP2A and CHMP3 (Agromayor and Martin-Serrano 2006; Tsang et al. 2006; Kyuuma et al. 2007; Ma et al. 2007). In the case of CHMP3, AMSH interacts with the open

conformation and requires a larger region than the predicted MIM C-terminal domain of CHMP3 (Fig. 30A). Mutagenesis of CHMP3 residues implicated in AMSH binding (K216, L217) does not affect the interaction of the CHMP3 C-terminus with its N-terminal core. Therefore the interaction with AMSH is probably not the driven force triggering the opening of CHMP3. The interaction site of AMSH with CHMP3 does not overlap with the CHMP1-AMSH binding site (McCullough et al. 2004; Zamborlini et al. 2006; Lata et al. 2008; Solomons et al. 2011). However, AMSH compete with Vps4 for the binding to the C-terminal regions of CHMP3, CHMP1A and CHMP1B (Agromayor and Martin-Serrano 2006; Solomons et al. 2011). Therefore, cargoes trapping probably needs to be fully completed before the Vps4 can be recruited to the ESCRT-III polymer.

AMSH and Hrs-STAM both interact directly with clathrin heavy chain and a model has been proposed where AMSH-STAM-Hrs trimeric complex interact with clathrin at the level of CCV. AMSH could deubiquitinate either the receptor or the Hrs-STAM complex reversing receptor internalization and downregulation. Moreover, AMSH may work as a bridge between the ESCRT-0 and ESCRT-III complexes, in that case, favoring endocytic trafficking to the lysosomes (McCullough et al. 2006; Urbe et al. 2006; Sierra et al. 2010). Finally, AMSH also interacts with Alix and is necessary for receptor degradation in the plant model *Arabidopsis thaliana* (Katsiarimpa et al. 2013; Katsiarimpa et al. 2014; Kalinowska et al. 2015).

The different role described for AMSH during EGFR trafficking point out putative opposite functions that may depend on other regulatory proteins or mechanisms favoring one role or the other. This regulators remain to be described.

4.4.1.2 UBPY

UBPY is a cysteine protease of the ubiquitin-specific protease family (Amerik and Hochstrasser 2004). Its catalytic activity is regulated by ubiquitination and auto-deubiquitination during EGF stimulation (Wu et al. 2004; Mizuno et al. 2005) on the one side and by ligand induced Src-phosphorylation (Alwan and van Leeuwen 2007) on the other side. UBPY catalytic activity can hydrolyze monoubiquitin linkage and it can also process K48 and K63 chains to lower denomination forms *in vitro* (Row et al. 2006). UBPY is recruited to the early endosomes after 10 min of EGF stimulation and its catalytic activity is necessary for its disassociation from the membranes (Rives et al. 2006).

UBPY interact with and deubiquitinates Ub-EGFR inhibiting (Mizuno et al. 2005; Mizuno et al. 2006; Alwan and van Leeuwen 2007) or in contrast, promoting

(Bowers et al. 2006; Row et al. 2006; Alwan and van Leeuwen 2007; Zhang et al. 2014) EGFR lysosomal degradation, depending on which step of the sorting is being concerned : i.e., the early step or later step (at the level of MVB), respectively. Over-expression of catalytic dead form of UBPY leads to enhanced activation and ubiquitination of EGFR with enhanced MAPK activation, enhanced EGFR expression levels and blockage of the lysosomal degradative products of the EGFR (Alwan and van Leeuwen 2007). However, such overexpression of a catalytically dead enzyme may have unspecific effects rending the interpretation of the physiological role of UBPY, which may activity either at the level of the PM or of the MVB might be tightly regulated in physiological conditions, difficult to determine.

From the literature, the following events can be proposed:

Early steps: UBPY binds and deubiquitinates Mono-ub-Eps15 similarly as USPX9: this function is discussed below (*section 4.4.1.3*) (Mizuno et al. 2006; Savio et al. 2016). However, the internalization of the EGFR is not affected by UBPY activity (Mizuno et al. 2005; Row et al. 2006; Row et al. 2007).

UBPY also directly interacts with the SH3 domain of the ESCRT-0 STAM (Fig. 30B), which stimulates its catalytic activity; however the endosomal localization of UBPY is not dependent of its interaction with the Hrs-STAM complex. UBPY deubiquitinates Hrs but not STAM, protecting the Hrs-STAM complex from proteasomal degradation (Kato et al. 2000; Mizuno et al. 2005; Mizuno et al. 2006; Row et al. 2006; Zhang et al. 2014).

Expression of the catalytic-inactive domain of UBPY has no effect on EGFR while expressing a full length catalytic-inactive form of UBPY blocks EGFR sorting, probably due to dominant blockade of the pathway. Therefore, the MIT containing N-term domain of UBPY may be required for UBPY localization at the endosomes. Moreover, UBPY catalytic activity would protect the receptors from degradation (Alwan and van Leeuwen 2007; Row et al. 2007).

The most conclusive results comes from silencing experiments: in the *Ubpy* knock-down cells, EGFR, Hrs and STAM are all retained in their ubiquitinated forms in the endosomes. STAM and Hrs levels are decreased, and lysosomal EGFR degradation is inhibited (Row et al. 2006). Therefore, EGFR needs to stay ubiquitinated to reach the late endosomal sorting point where it needs to be deubiquitinated to enter the MVB pathway. Both steps would be tightly controlled by UBPY (Mizuno et al. 2005; Row et al. 2006; Alwan and van Leeuwen 2007; Row et al. 2007).

Thus, as in the case of AMSH, UBPY, can have opposite functions in EGFR degradation versus stabilization depending on its subcellular localization: at the level of early endosomes versus late endosome/MVB.

Later Steps: In yeast, Doa4 (orthologue of UBPY) associates with ESCRT components: Bro1 (Alix) recruits Doa4 to the endosomal membranes where Doa4 deubiquitinates cargoes before their internalization into the ILVs of the MVBs (Amerik et al. 2000; Amerik and Hochstrasser 2004; Luhtala and Odorizzi 2004). In humans the interaction of UBPY with Alix displaces STAM and allows the recruitment of CHMP4B. This interaction also permits the deubiquitination of EGFR by UBPY (Ali et al. 2013).

The MIT domain of UBPY interacts *in vitro* with several ESCRT-III CHMP proteins: CHMP1A, CHMP1B, CHMP4C and CHMP7. In HEK293T and Hela cells full length UBPY interacts with CHMP1A, CHMP1B and CHMP7, but not with CHMP3, in a MIT dependent manner. The MIT domain is actually strictly necessary for UBPY localization to the endosomal membranes. Therefore, the interaction between the MIT domain of UBPY and the ESCRT-III CHMP proteins triggers UBPY endosomal localization and regulates the substrate specificity of the enzyme. In contrast, UBPY interaction with STAM is necessary for STAM stabilization and EGFR degradation and this interaction activates the catalytic activity of UBPY Hrs. How these interactions are regulated by EGF signaling is not yet described (Row et al. 2007).

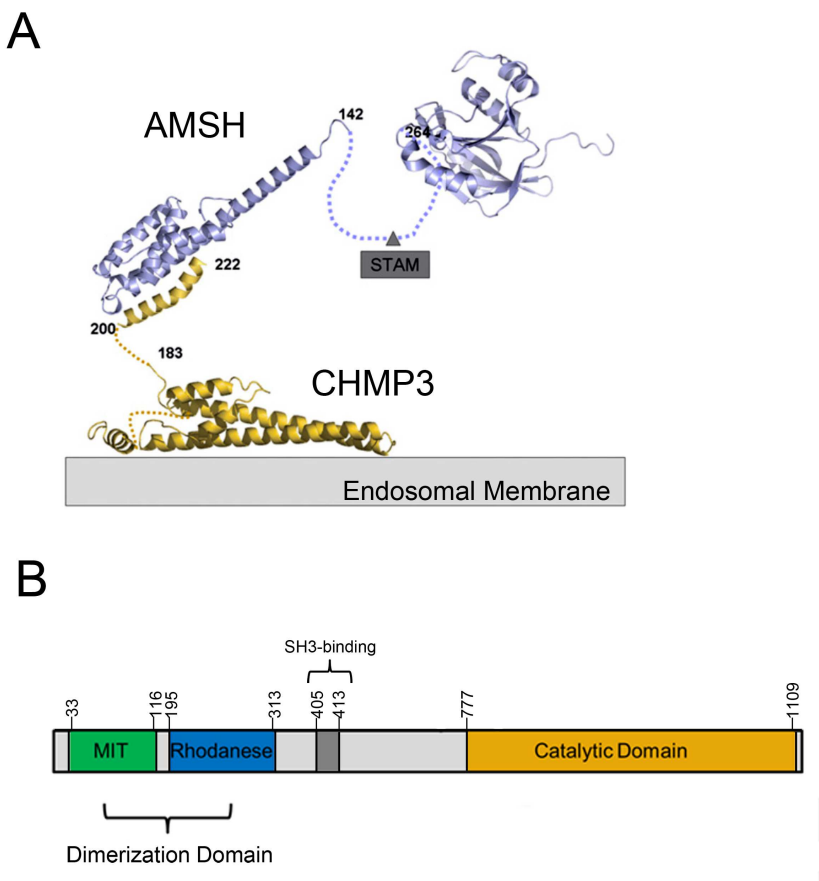


Figure 30: AMSH and UBPY. A: Molecular model of AMSH in complex with membrane-associated CHMP3. Modified from (Solomons et al. 2011). **B:** Schematic diagram of the domain structure of UBPY. The USP catalytic domain, the dimerization domain, the SH3 domain which interacts with STAM and the MIT domain which interact with the ESCRT-III proteins are labeled.

4.4.1.3 Others DUBs

USP18: USP18 catalytic activity specifically increases the expression levels of EGFR by 50-80% by inducing EGFR mRNA translation (Duex and Sorkin 2009).

Cezanne-1: This ubiquitin specific protease interacts and deubiquitinates the EGFR inhibiting its lysosomal degradation in both KB and Hela cells. Cezanne-1 function in EGFR stability and signaling require both its catalytic and its ubiquitin-binding domains as well as the transphosphorylation of Cezanne-1 by activated EGFR. Cezanne-1 also interacts with the E3 ligases AIP4 and Nedd4 that are both implicated in the regulation of the EGFR trafficking (Pareja et al. 2012).

USP2 would associate and deubiquitinate the EGFR at early endosomes, thus increasing the recycling ratios of EGFR and enhancing the activation of downstream signaling upon EGF stimulation (Liu et al. 2013).

USP9X: Eps15 monoubiquitination upon EGF stimulation inhibits its function by intramolecular autoinhibition (Klapisz et al. 2002; Polo et al. 2002) and delays EGFR internalization and degradation (Fallon et al. 2006; van Bergen En Henegouwen 2009). Deubiquitination of Eps15 by USP9X stimulates EGFR degradation (Savio et al. 2016). The authors propose that ubiquitination and deubiquitination cycles of endocytic adaptor proteins are required for EGFR trafficking at least in conditions of low EGF concentration. Actually, the idea that ubiquitination/deubiquitination cycles are important to govern many steps of the endocytic process makes sense and is an attractive hypothesis to explain many controversial results in the field; Already proposed and reviewed in (Madshus and Stang 2009).

5. ENDOCTYOSIS and DEVELOMENTAL SIGNALS

5.1 Regulation of Signaling by Endocytosis

5.1.1 Introduction

According to the Royal English Academy, the general definition of Signaling is "the action of generating or transmitting signals". In biology, a signal is considered a physical entity, such as a chemical or an electromagnetic wave, that contains the information to be transmitted and that activates cell receptors which in turn elicits a specific response.

Four main types of signaling exist. The **autocrine** signaling relating to self-stimulation, through the concomitant production of both a signal and its specific receptor. The **jusxtacrine** signaling where a cell places a specific signal called ligand on the surface of its membrane, and subsequently another cell can bind it with an appropriate cell surface receptor or cell adhesion molecule (an important example is the Notch signaling). The **paracrine** signaling, in which a cell produces a signal to activate receptors in nearby cells (like the FGF pathway, the Jak-STAT pathways, the Wnt pathway, the Hedgehog (HH) pathway and the TGF-β superfamily of signaling pathways and finally, the RTK receptors). The last type is the **endocrine** signaling, where the signals produced by a cell travel long distances through the circulatory system and activate receptors in cells far away (hormones communication).

Signaling systems are tightly regulated so that signals only act in the cells that are meant to interpret them at the appropriate time and at the right concentration. This implies that those cells present the right amount of receptors and transduction components in the proper status (Sorkin and Von Zastrow 2002; Piddini and Vincent 2003).

Endocytosis provides spatial and temporal dimensions to signaling and can work in homeostatic loops to prevent excessive ligand-induced activation of downstream effectors. Endocytosis is implicated in the regulation of most of the signaling pathways previously named and particularly in the Notch pathway (Parks et al. 2000; Brou 2009; Chastagner and Brou 2014); The FGFR pathway (Wiedlocha

and Sorensen 2004) the HH pathway (Incardona et al. 2000; Incardona et al. 2002; Matusek et al. 2014); the Wnt pathway (Blitzer and Nusse 2006); The TGF- β superfamily pathway (Penheiter et al. 2002) ; and the EGFR pathway (Sorkin and Von Zastrow 2002). In all the steps of the signaling process, from the producing to the receiving cell, endocytosis contributes to the formation of morphogen gradients; modulates interactions between signaling molecules and their receptors; regulates receptor presentation at the cell surface; controls activation of signaling cascades; provides a physical adjustable platform for the transduction of the signaling to take place; regulates the sorting of the internalized receptors to degradation or recycling; regulates the termination of the signaling and degradation of internalized ligands,…

Differences in the endocytic trafficking pathways can also distinguish the physiological regulation of otherwise similar receptors or ligands subtypes. Actually, each signal takes its own endocytic strategy making very difficult to describe common intertwining molecular mechanisms for trafficking networks during signaling.

In this chapter, a short overview of how the subcellular localization of protein complexes and spatial distribution of signaling components add complexity to a specific phenotypic responses will be addressed. For the purpose of this study the specific mechanisms and consequences for development of the endocytosis of Notch or EGFR signaling pathways has been chosen.

5.1.2 Ligand Delivery

Most of the signaling ligands are lipid-associated or lipid modified (Porter et al. 1996). From the producing cell, a membrane ligand can be released by direct disengagement from the membrane, or in membrane carriers such as argosomes or exosomes. Ligands may also travel through the endocytic pathway of neighboring cells before being released in the extracellular space in a process called planar transcytosis. Exosomes carriers have been notably implicated in the Notch, the HH and Wnt signaling pathways (Matusek et al. 2014; D'Angelo et al. 2015; McGough and Vincent 2016).

5.1.3 Ligand Gradient Control

Morphogen gradients are hypothesized to be formed by two main mechanisms: passive diffusion and vesicle-mediated transport also called planar

transcytosis (Strigini and Cohen 1999; Teleman et al. 2001; Gonzalez-Gaitan 2003a) (Fig. 31).

In these processes, endocytosis efficiently removes signaling molecules from the extracellular matrix and therefore controls the extracellular concentration. In the case of transcytosis, the range and slope of the ligand gradient is determined by the relative rates between recycling compared to degradation of the ligand in the receiving cells. This is the case for Dpp and Wg (*Drosophila* Wnt) signaling (Entchev et al. 2000). (Dubois et al. 2001; Srinivasan et al. 2002; Gonzalez-Gaitan 2003a).

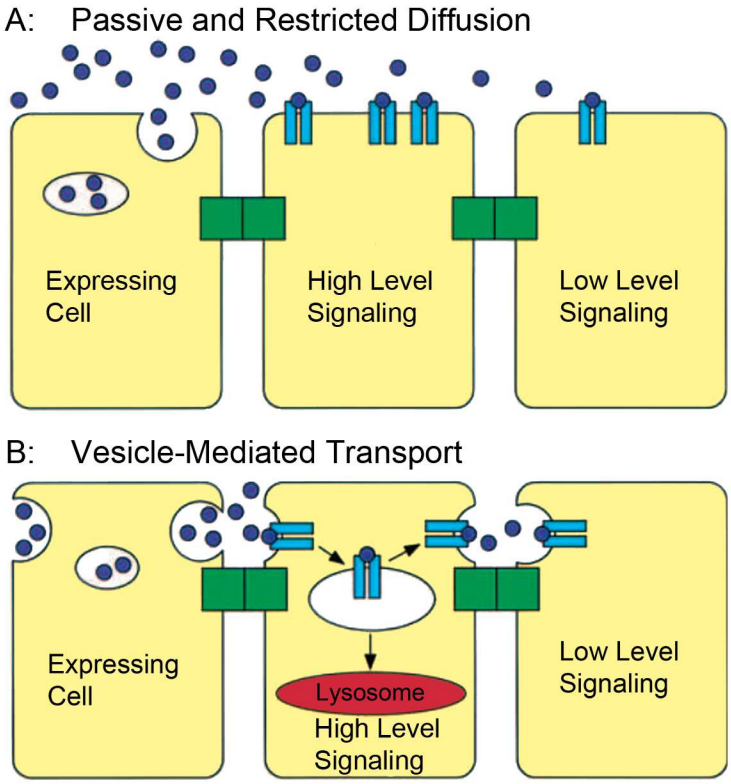


Figure 31. Models of morphogen gradient formation. A: The diffusion model of gradient formation. Diffusion may be passive leading to rapid gradient formation or restricted diffusion attributable to morphogen interaction with extracellular matrix proteins, membrane proteins, or membrane lipids. **B:** The vesicle-mediated model of gradient formation or transcytosis. Upon release from the expressing cell the morphogen bound to receptors on neighboring cells and internalized. There it can be degraded or recycled to the membrane surface and released again regulating the relative ratio of recycling to lysosomal degradation will determine the shape of the gradient. Modified from (Seto et al. 2002).

5.1.4 Signal Transduction

As explained in the *section 4.3.3* and *2.2.1* two different routes of internalization to the endosomal pathways exist mediating recycling *versus* degradation of the receptors. However, the same route used for receptor degradation in one signaling pathway can be used for recycling/signaling by other receptors. In addition, the intracellular trafficking routes are consider more as networks where the signal can be modulated by accelerating or braking the speed of the arrival to the final destination of the signaling complexes. Moreover, signaling itself can influence the architecture of the network. Adding a level of complexity, each signal takes its own network strategy making almost impossible to describe a unifying theory for trafficking during signaling. Reviewed in (Gonzalez-Gaitan 2008). One of the most important point to highlight is the **capacity of the endosomes to act as signaling platforms.** Historically, trafficking through the endocytic pathway has been thought as a mechanism to "turn off" or inactivate the stimulated receptor; however, endocytic vesicles have been also rather recognized as platforms for signaling events (Ceresa and Schmid 2000; Leof 2000; Alexia et al. 2013; Ceresa and Peterson 2014). In these cases, signaling event can take place while the receptor-ligand complex is *en route* through different endosomal compartments and two types of endosomal signaling have been proposed. Reviewed in (Sorkin and von Zastrow 2009) and (Gonzalez-Gaitan 2003b)):

(1) *Sustained signaling in endosomes:* The signal that it is initiated in the plasma membrane continues during the endosomal traffic of the signaling complex.
(2) *Endosome-specific signaling:* A different signaling event occurs in the endosome thanks to the assembly of specific signaling complexes that does not occur at the membrane. TGFβ, GPCR, RTK, Notch, TNF, Toll-like receptor have this kind of signaling.

The other general mechanism of endosomal sorting have been described in the *section 2.2.3 Mechanisms of Endosomal Sorting*. The sorting of the EGFR as a model for endosomal receptor sorting has been described in the *section 4.3.4 Role of Ubiquitination in EGFR Sorting: Degradation or Recycling*.

I just wish to remind and shortly point out here the implications of this sorting in the down-regulation or termination of downstream signals:

Signal termination: endocytosis can down-regulate or terminate the unitary signal (coming from a unique signaling complex) by inducing ligand-receptor dissociation through the delivery of the ILVs containing the receptor-ligand complex to the

lysosomes (Sorkin and Von Zastrow 2002) or by separating the activated receptors from their plasma-membrane substrates or mediators during the internalization process, -this is notably the case of the PI3K signaling in the EGFR signaling pathway (Haugh 2002a; Haugh 2002b; Haugh and Meyer 2002), or by separating the activated receptors from their endosomal or cytosolic substrates or mediators during ILVs formation (Sorkin and Von Zastrow 2002).

Sorting: the first sorting decision happens at the membrane and consists in triggering or not the internalization process (terminating the signal when it occurs from the PM). This decision highly depends on the linkage of ubiquitin to the cargoes as described in the *section 4.3.3 Role of the Ubiquitination of EGFR at the Plasma Membrane: CME or CIE*.

The second sorting decision happens in the endosomal membrane: recycling or degradation? In the case of recycling event, the cell will recover the signal computation ability. In the case of degradation, the computational capacity diminished over time.

5.2 Regulation of the EGFR Signaling by Endocytosis

The regulation of the quantity and physical localization of the EGFR or of the EGF-EGFR complex by the endocytosis process and the ubiquitin system in particular has been described in the *sections 4.3* and *4.4*. Here, I will point out how endocytosis can trigger differences in the nature of downstream signals activated.

Activated EGFR can engage at least six biochemical pathways also shared by other receptors, leading to cell fate decisions. Regulation of these downstream pathways by the endocytosis process belongs to the "early regulatory loops" which include protein translocations to cell compartments and/or post-translational modifications. On the other side, the "late regulatory loop" depends on the transcriptional response to EGF. (Avraham and Yarden 2011). Two kinds of endocytosis dependent regulation of downstream signals exist in the case of the EGFR: first, a down-regulation through lysosomal degradation; second, a signal induction through endocytosis by bringing the active receptor to specific downstream targets within the cell.

Activated EGFR can signal from the PM or from the endosomes, triggering different signals (Sorkin and Von Zastrow 2002; Miaczynska et al. 2004b; Miaczynska 2013). Indeed, the EGFRs can remain ligand bound, phosphorylated

and activated on endosomal membranes, until their internalization into the ILVs at the MVB (Sorkin and Von Zastrow 2002).

For example, EGFR cellular internalization is necessary for the activation of the PKB/AKT pathway but not for the activation of the MAPK pathway (Goh et al. 2010). However, other studies show that activation of the PKB/AKT pathway occurs mostly from the plasma membrane (Sorkin and von Zastrow 2009) and that activation of the MAPK pathway happens from endosomes where the EGFR binds its effector Grb2 and activate the MAPK pathway (Sorkin et al. 2000; Teis et al. 2006; Sorkin and von Zastrow 2009). Therefore, endocytosis of the EGFR is probably necessary for signaling after the MEK1 activation and before activation of MAPK (See Figure 25). Activated MAPK can be imported to the nucleus to activate the mitogen response. By association with β-Arrestins, activated MAPK can remain in the endosomes and keep phosphorylating cytosolic targets like the JNK3 (McDonald et al. 2000). The contribution of signaling from the EGFR to the MAPK in endosomes is certainly extremely important and may depend on the speed of targeting the EGFR to the ILVs. Differences in the results reported so far may reflect cell types or experimental conditions dependent requirement of various endocytic steps in EGFR signaling.

As another specific example: specialized signaling endosomes that are Rab5-positive and EEA1-negative contain the adaptor proteins APPL which can interact directly to EGFR (Miaczynska et al. 2004a). APPL affects ERK1, ERK2 and Akt/GSK3 or TOR signaling in mammals. It is proposed that APPL delays the maturation to EEA1 positive endosomes of the EGFR-EGF containing endosome, thus delaying their degradation and extending downstream signaling (Zoncu et al. 2009).

5.3 Regulation of Endocytosis by EGFR Signaling

EGFR activity itself regulates the internalization of some PM receptors through the phosphorylation of downstream target proteins (Glenney et al. 1988; Lamaze and Schmid 1995). Indeed, a large number of proteins implicated in the regulation of endocytosis are tyrosine kinases phosphorylated by RTKs including the EGFR. This feature was notably pointed out in a genomic screen setup for finding kinases implicated in endocytosis that also revealed that most of the identified kinases where implicated in only one type of endocytosis: CME or CIE and in the cases of kinases that would regulate both CME and CIE, they would have

opposing effects unveiling a vast regulation of endocytosis by signaling (Pelkmans and Zerial 2005; Polo and Di Fiore 2006).

Regulation of the internalization machinery: EGFR activation mediates the *de novo* clathrin coated pits formation by activating the Srk kinase which in turn phosphorylates the clathrin heavy chain which controls its assembly (Wilde et al. 1999). Moreover, Eps15 is recruited to clathrin-coated pits in response to EGFR activation, and EGFR-mediated phosphorylation of Eps15 is required for ligand induced internalization of EGFR (Confalonieri et al. 2000). In neurons, depolarization of the pre-synaptic terminal activates many phosphatases which dephosphorylate numerous endocytic proteins including dynamin and amphiphysin enhancing CME of different membrane proteins (Marks and McMahon 1998).

Regulation of the sorting machinery: EGFR activation induces Rab5-GDP complex formation by the p38 MAP kinase which accelerate endocytosis and promotes internalization of EGFR. ERK1 and ERK2 signaling then regulate endosomal maturation and cargoes degradation (Teis et al. 2006). Activation of PKA accelerates Rab4-dependent recycling. Finally, the regulation of Rab proteins during signaling guides the overall organization of subcellular compartments of the cells (Gurkan et al. 2005; Ceresa 2006; Ceresa and Bahr 2006; Stenmark 2009; Wandinger-Ness and Zerial 2014).

Regulation of the ubiquitination machinery: Many ligands regulate the activity and localization of E3 ubiquitin ligases Nedd4 or Cbl (Hicke 2001), UBE4B (Sirisaengtaksin et al. 2014), Nrdp1 (Qiu and Goldberg 2002; Cao et al. 2007) and probably also AIP4/ITCH, as well as the deubiquitinating enzymes like AMSH or UBPY (Alwan and van Leeuwen 2007). Sprouty 2, Tbc1d3, Cool-1, Src and NEDD4-1 regulate Cbl activity on EGFR traffic through the binding to the receptor or the ligase during signaling (Wong et al. 2002 (Bao, 2003 #723; Bao et al. 2003; Rubin et al. 2003; Wu et al. 2003; Feng et al. 2006; Wainszelbaum et al. 2008). How the activity of this proteins is regulated by signaling is still poorly understood. In *Drosophila* Myopic (Faucz et al.), the homologous of mammalian tyrosine phosphatase Alix/HD-PTP (which associated with ESCRT-III) interacts with Cbl and regulates EGFR degradation but the exact mechanisms of this regulation are unknown (Miura et al. 2008).

Regulation of the receptor: multiple protein tyrosine-specific phosphatases (PTPs) reverse tyrosine phosphorylation on EGFR and other RTKs. One example is the PTP1B which has been implicated in EGFR signaling (Haj et al. 2002) by dephosphorylation of EGFR just before inward invagination into the MVB or

alternatively dephosphorylating signal transducing adapter molecule 2 (STAM2), a component of ESCRT-0 (Stuible et al. 2010). A motor protein of the Kinesin-3 family, KIF16B, anchors PTPD1 to early endosomes promoting EGFR recycling by regulating microtubule-dependent motility (Hoepfner et al. 2005) and PKC-dependent phosphorylation of T654 of the EGFR diverts the EGFR-EGF complex to recycling (Bao et al. 2000).

5.4 EGFR Signaling in *Drosophila* Wing Development

The adult *Drosophila* wing arise from primordial tissue known as wing imaginal disc (WID). In the larva, developmental signal induce the WID cells to proliferate and induce compartmentalization, specification and patterning.

For a general scheme of the morphology of the *Drosophila* wing development and the pattern of expression of the different signal see *Annex II*. Briefly, the WID is subdivided into anteroposterior (AP), dorsoventral (DV) and wing-notum (limb-body wall) primordia. The AP and DV subdivisions are developmental compartments that are established via the heritable activation of the genes *engrailed* (en) and *apterous* (ap). The activation or absence of expression of these genes generate cellular interfaces called "boundaries". Boundaries act as barrier to cell mixing and are the source of morphogens that organize the development of both compartments.

Early in development, Vein (Vn), one of three known ligands for the EGFR, is expressed in the dorsoproximal WID and activates EGFR. EGFR signaling activates asymmetrical *ap* expression (Wang et al. 2000; Zecca and Struhl 2002a; Zecca and Struhl 2002b). The resulting asymmetrical Ap expression segregates the WID into D (ap-ON,) and V (ap-OFF,) compartments. The Vn-EGFR signaling, and therefore the expression of Ap, is repressed in the ventrodistal region of the WID by the Wg signaling gradient (Bieli et al. 2015) (Fig.32 Larva I).

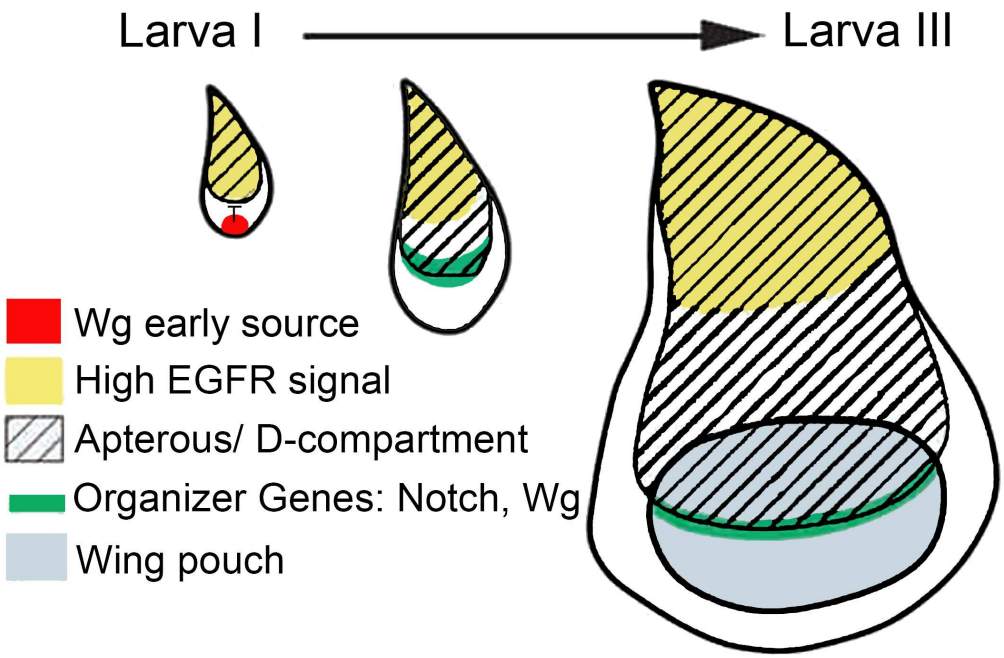


Figure 32: Control of WID development by EGFR signaling from Larva I to Larva III. Modified from (Zecca and Struhl 2002a; Zecca and Struhl 2002b).

As the disc grows, the DV boundary moves ventrally, beyond the range of the instructive EGFR signal. The ap-ON ap-OFF interface must be located in a region of low EGFR signaling to allow D and V compartment cells to interact and initiate a positive auto-feedback loop of Delta/Notch and Serrate/Notch signaling that drives the reciprocal induction of Wg and Vg expression on both sides of the DV compartment boundary which in turn control subsequent wing development. (Zecca and Struhl 2002a; Bieli et al. 2015) (See next section and Fig.32 Larva III).

Proper intracellular trafficking of Vn-EGFR is essential in this process. Little perturbations of the trafficking machinery can produce enormous impact in the adult structures. Some ESCRT members have been described to regulate EGFR signaling in *Drosophila* wing development. The EGFR degradation upon signaling is regulated by the ESCRT-0 Hrs and the ESCRT-III Chmp1 besides others. ESCRT notably play a major role during development in receptors endocytosis and regulation of downstream signals as well in the formation of long-range gradient of ligand by exocytosis/transcytosis (Lloyd et al. 2002; Valentine et al. 2014).

5.5 Notch Signaling in *Drosophila* Wing Development.

Introduction to the Notch pathway:

In *Drosophila* the Notch encoding gene is present on the X chromosome. Notch is a transmembrane dimeric receptor and Delta is a transmembrane ligand of Notch. Fringe and Serrate are other Notch ligands (Ju et al. 2000). The Delta-Notch complex is a juxtacrine signaling pathway that was first described in *Drosophila* and is widely conserved in metazoans. Delta-Notch is a major player during stem cell maintenance, cell fate specification and boundaries formation by producing lateral inhibition and asymmetric cell division in most of the *Drosophila* tissue/organs (Greenwald 1998; Artavanis-Tsakonas et al. 1999; Gonzalez-Gaitan 2003a; Carlson and Conboy 2007; Aoyama et al. 2013; Guo and Ohlstein 2015; Ahmad 2017; Harris 2017; Januschke and Wodarz 2017; Kojima 2017; Ku and Sun 2017; Siebel and Lendahl 2017; Trylinski et al. 2017; Zhai et al. 2017). The Notch receptor contains EGF like motifs that interact with its ligands Delta and Serrate containing EGF binding domains. In the absence of activation, Notch is constantly internalized and degraded by the conventional lysosomal degradative pathway. Activation of Notch leads to two proteolytic cleavage, the first by the protease ADAM in the membrane and the second by the γ-secretase in the endosomal membrane (Brou et al. 2000). After that, the Notch intracellular domain is translocated to the nucleus where it acts in complex with Suppressor of Hairless (Su(H)) to trigger a specific developmental program (Weinmaster 1997; Greenwald 1998).

Notch Pathway in Drosophila Wing development: D/V boundary

To see previous developmental step during *Drosophila* wing development see *Annex II*.

During wing development, the ligands of Notch are expressed in a Fringe-dependent compartment specific manner. Ap activates the expression of Fringe and Serrate in the dorsal compartment wich in turn activate Notch (Diaz-Benjumea and Cohen 1995) whereas Delta is the signal expressed by the ventral cells to activate Notch. Thus, although Notch is expressed uniformly in the wing disc, it is only activated at the D/V border (de Celis et al. 1996)). Fringe is expressed in the dorsal tissue and inhibits effectiveness of Serrate in dorsal tissue and potentiates effectiveness of Delta in ventral tissue (Wu and Rao 1999), (Fleming et al. 1997a; Fleming et al. 1997b). There is a feedback loop between Delta-Notch and Wg to Fz-2 that results in the restriction of Notch activation only in the dorsoventral boundary

and only there, the expression of Wg is activated (de Celis and Bray 1997; de Celis et al. 1997). Activation of Notch at the D/V border leads to expression of Wg, vestigial (vg) and other genes specific for the D/V border, and promotes the outgrowth of the wing (de Celis et al. 1996) (Fig. 33).

Regulation of the Notch Pathway by Endocytosis

The fact that endocytosis is regulating Notch in neuron asymmetric division was first showed by (Seugnet et al. 1997). Today, many steps of the Delta-Notch pathway are known to be regulated by endocytosis allowing to generate asymmetric cell division during development (Hurlbut et al. 2007; Aoyama et al. 2013; Guo and Ohlstein 2015):

***Delta endocytosis*:** the endocytosis of the Notch extracellular domain (NotchECD) by the Delta expressing cell is necessary to activate Notch signaling in the Notch expressing cells (Weinmaster 1997; Greenwald 1998). Delta ubiquitination by the E3 ligase Neuralized (Neur) promotes the internalization of the Delta-NotchECD complex. It was proposed that the mechanical force exerted by its internalization helps Notch cleavage or alternatively that Neur acts in the Notch presenting cell to degrade Delta, thus releasing Notch from Delta *cis*-inhibition (Lai et al. 2001; Lai and Rubin 2001b; Lai and Rubin 2001a). Once internalized, Delta can pass by the Rab11 recycling endosomes and go back to the membrane. This mechanism can be inhibited in a Sec15-dependent manner (Emery et al. 2005).

***Notch endocytosis*:** in *Drosophila,* Notch endocytosis regulates Notch activity by modulating its availability at the surface of responding cells and allowing for its translocation into the cells (de Celis et al. 1997; Fleming et al. 1997b; Artavanis-Tsakonas et al. 1999; Emery et al. 2005; Brou 2009; Chastagner and Brou 2014; Loubery and Gonzalez-Gaitan 2014; Harris 2017; Januschke and Wodarz 2017). The trafficking of the Notch receptor is regulated by its ubiquitination by the E3 Suppressor of Deltex/Itch/AIP4 which induces Notch lysosomal degradation (Qiu et al. 2000; Chastagner et al. 2008).

As another level of regulation, Numb is localized in the endosomal membrane and can interact with Notch and α-adaptins, bridging them together and trapping Notch in the endosome. This reduces the level of Notch at the plasma membrane and its availability to bind Delta (Weinmaster 1997; Amerik et al. 2000; Berdnik et al. 2002; Kojima 2017). Sanpodo, a protein necessary for Notch signaling at the plasma membrane is also trapped by Numb in the endosomes of certain cells,

inhibiting Notch signaling (Hutterer and Knoblich 2005; Couturier et al. 2013; Upadhyay et al. 2013; Couturier et al. 2014)). In addition, the Deltex E3 ligase monoubiquitinates activated and cleaved Notch Intracellular Domain (NotchICD) and promotes its accumulation in the membrane of the MVB by inhibiting its internalization into the ILV (Yamada et al. 2011). In the MVB membrane, NotchICD is cleaved by the γ-secretases and signaling is triggered (Gupta-Rossi et al. 2004; De Kloe and De Strooper 2014).

Members of each family of the ESCRT machinery act as modulators of Notch trafficking (Hori et al. 2012; Aoyama et al. 2013; Schneider et al. 2013; Juan and Furthauer 2015). In cells depleted for the ESCRT-0 Hrs or for upstream endosomal components, Notch accumulates without major effects on Notch signaling (Lloyd et al. 2002; Jekely and Rorth 2003). However in the *Drosophila* wing cells depleted of Hrs downstream components like the ESCRT-I Tsg101, ESCRT-III Vps22, Vps25 or ESCRT-III Vps32 (CHMP4) display accumulation and ectopic activation of Notch resulting in overgrowth phenotypes. Reviewed in (Moberg et al. 2005; Thompson et al. 2005; Wilkin et al. 2008; Brou 2009; Vaccari et al. 2009). This over-activation may result from the same mechanism of MVB membrane trapping proposed for Deltex (Furthauer and Gonzalez-Gaitan 2009).

On the other hand, in mammalian cells, ligand-independent activation of Notch is not detected and, since the mutants of the ESCRT machinery are in most of the cases embryonic lethal, the implication of the ESCRT machinery in the Notch pathway are still challenging to analyze (Brou 2009)

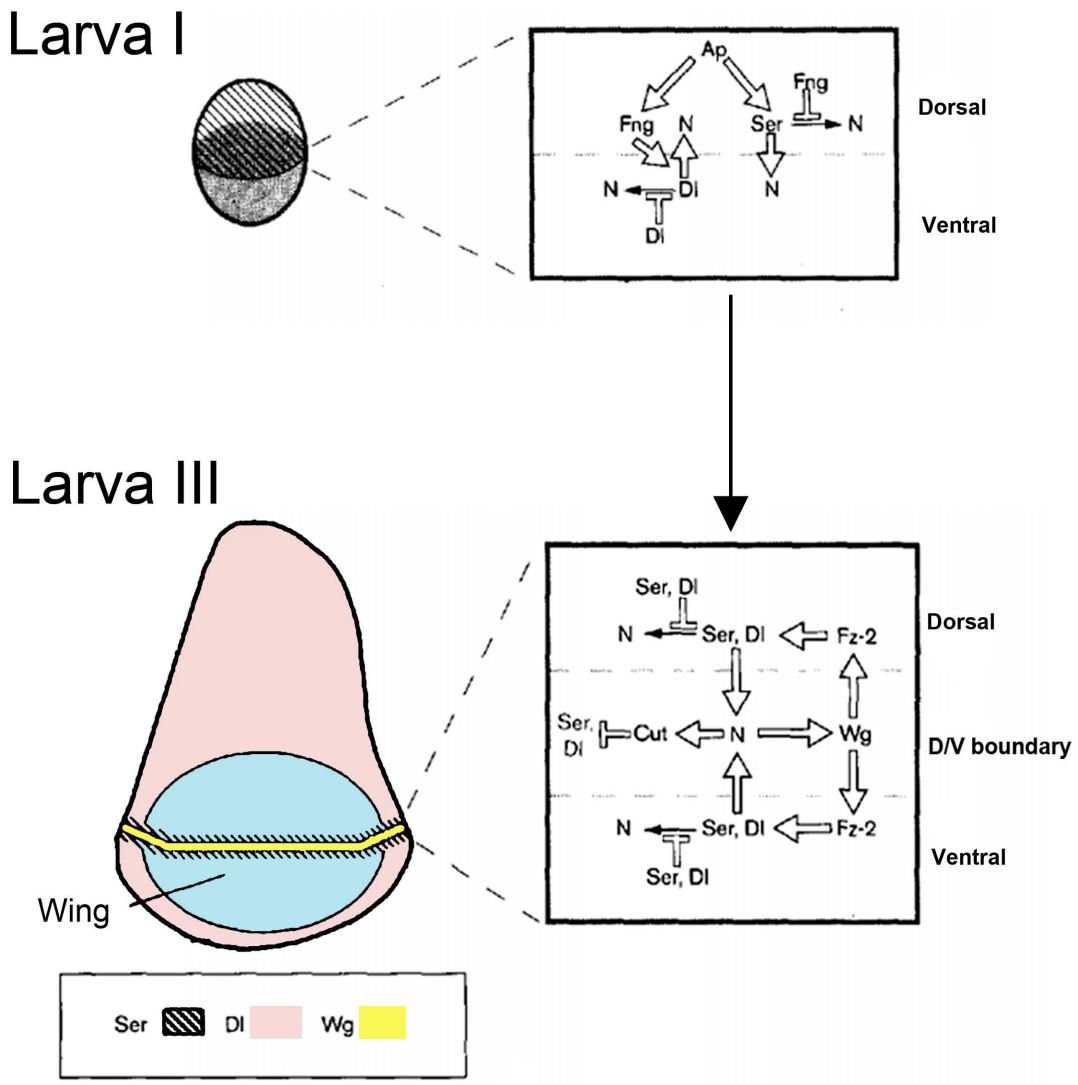


Figure 33: Simplify Delta-Notch dependent D/V boundary formation in the Drosophila wing imaginal disc: Larva I: Signaling events of the Delta-Notch pathway from at the larval stage 1. **Larva III:** Signaling events of the Delta-Notch pathway from at the larval stage 3. Ser: Serrate; Dl: Delta; Wg: Wingless; N: Notch; Fz-2: Frizzled2; Fng: Fringe.

II. Results

Unveiling Ubiquitination as a New Regulatory Mechanism of the ESCRT-III Protein CHMP1B

1. History and Environment of the Project.

Marie-Odile Fauvarque's group carry out genetic and chemogenomic strategies for the identification and the study of the function of proteins and chemical molecules in intracellular signaling.

In particular, one part of the team conducts research to provide fundamental knowledge on the ubiquitin system function in cell signaling with a strong focus on DUBs controlling inflammation and innate immune response or tumorigenesis in human cells. In humans, these DUBs represent potential biological targets for the screening and selection of small chemical molecules usable as research tools or as therapeutic entities. Conservation of signaling pathways and regulatory processes during evolution makes relevant the use of the fruit flies *Drosophila melanogaster* as a model system for functional studies of the corresponding DUB in a living organism.

In this context, my thesis work aimed at the functional characterization of a protein complex formed by CHMP1B and UBPY during endocytosis and cell signaling.

The interaction of this two proteins had been first reported in both a yeast two hybrid system and in human cells (Row et al. 2007). In this study, the author show that the MIT domain of UBPY is necessary for UBPY localization to the endosomal membranes as well as for STAM stabilization and EGFR degradation. However, no further result about the function of this interaction has been reported since then.

As part of the Labex GRAL (Grenoble Alliance for Integrated Structural & Cell Biology), Dr. C. Aguilar-Gurrieri, recruited as a post-doctoral researcher in Pr. W. Weissenhorn's group- and myself have taken over a project aiming at understanding and characterizing the structure and the function of the interaction between CHMP1B and UBPY. On the one hand, Dr. C. Aguilar-Gurrieri has employed biochemical and structural biology approaches for the in-depth study of

the interaction between the two partner proteins and the screening of molecules that could disrupt it. To this end, among other work not presented in this thesis manuscript, she did setup with the help of Dr. E. Soleilhac, the constructs that allowed the visualization of the complex by Venus complementation (BiFC) in living cells. On the other hand, I have used biochemical and cellular biology methodologies as well as transgenesis in *Drosophila* fruit flies to study the inter-regulatory relationships between the two proteins and their role in endocytosis and cell signaling. I took advantage of some constructs previously obtained by Dr. A.-C. Jacomin (notably in Fig. R1) and of the help provided by Dr. E. Taillebourg, particularly for the statistical analysis of confocal images (notably in Fig. R4), as well as the technical help of Nolwenn Miguet during the purification of CHMP1B oligomers by using sucrose gradients that I performed in Pr. W. Weissenhorn's laboratory.

My thesis work notably demonstrates the previously uncharacterized direct regulation of the ESCRT-III member CHMP1B by the linkage of ubiquitin that is strongly induced in response to EGF or pro-inflammatory cytokines and points out a new mechanism of ubiquitin dependent regulation of protein oligomerization controlled by UBPY. These findings are reinforced by the fact that CHMP1B ubiquitination is required for proper EGFR degradation in human cells and *Drosophila* wing development.

In summary, the results presented here support an emerging concept where ubiquitin linkage plays a crucial role in controlling protein oligomerization.

2. Short Recall of the Scientific Background

The linkage of ubiquitin moieties to plasma membrane receptors play a crucial role in signal activation and termination by inducing their internalization and subsequent trafficking and sorting along the endocytic pathway. From early endosomes, the receptors can either be sent back to the plasma membrane through recycling vesicles or directed for degradation via the multivesicular bodies (MVB) that eventually fuse with the lysosomes (Katzmann et al. 2001; Longva et al. 2002; Madshus and Stang 2009; Stringer and Piper 2011; Wright et al. 2011; MacDonald et al. 2012; Goh and Sorkin 2013). In MVBs, sorting of membrane cargoes requires the conserved ESCRT (Endosomal Sorting Complex Required for Transport) machinery which recognizes ubiquitinated receptors and drives the membrane deformation allowing the formation of intraluminal vesicles (ILVs) containing the receptors. The ESCRT machinery also plays essential function in other processes

requiring membrane remodeling like cytokinesis, some enveloped virus budding, nuclear pore quality control and reformation and plasma membrane repair (Agromayor et al. 2009; Hurley and Hanson 2010; Weissenhorn et al. 2013; Olmos et al. 2015; Vietri et al. 2015).

The ESCRT machinery consists in five complexes called ESCRT-0, I, II, III and the VPS4 complex that are all required for the MVB biogenesis (Henne et al. 2011). The ESCRT-0, I and II cluster ubiquitinated cargoes and initiate membrane bending while the ESCRT-III is responsible for the membrane fission-fusion event that releases the ILVs into the lumen of the MVB (Wollert and Hurley 2010).

The human ESCRT-III family is composed of 11 proteins termed CHarged Multivesicular Proteins (CHMP1A, B, CHMP2A, B, CHMP3, CHMP4A, B, C, CHMP5, CHMP6 and IST1) (McCullough et al. 2013) which are recruited to the membranes (Saksena et al. 2009). These CHMPs are small helical assemblies containing a core helical hairpin (Muziol et al. 2006; Xiao et al. 2009) and a C-terminal regulatory region (Lata et al. 2008a). They can be found in a closed auto-inhibited conformation or in an open activated conformation when the C-terminal region is displaced away from the core helical hairpin (Shim et al. 2007; Lata et al. 2008a; Bajorek et al. 2009). Activated ESCRT-III proteins can polymerize and form either homo- or heteromeric spiral structures *in vitro* and *in vivo* (Ghazi-Tabatabai et al. 2008; Hanson et al. 2008; Lata et al. 2008b; Bajorek et al. 2009; Bodon et al. 2011; Guizetti et al. 2011; Shen et al. 2014). For instance, activation of CHMP1B causes a modification of the structure of the flexible region between the two α-helices, α2 and α3, leading to a single elongated α-helix. CHMP1B polymers are stabilized by swapping of the C-terminal α-helices 4 and 5 (McCullough et al. 2015). A similar structure has been observed for of the activated yeast Snf7 protein (orthologous to CHMP4), indicating common principles for activation and polymerization of the CHMPs (Tang et al. 2015).

The regulatory C-terminal α-helix 6 contains one or two MIT interacting motifs (MIM) that recruit ESCRT-III partners via their Microtubule Interacting and Trafficking (MIT) domain such as ATPase VPS4 (Obita et al. 2007; Stuchell-Brereton et al. 2007) or the DeUBiquitinating (DUBs) enzymes : Ubiquitin Specific Protease 8 (USP8/UBPY) and Associated Molecule with SH3 domain of STAM (AMSH). Both, UBPY and AMSH are implicated in the regulation of endosomal trafficking and interact with various members of the ESCRT-III family (Agromayor and Martin-Serrano 2006; McCullough et al. 2006; Kyuuma et al. 2007; Ma et al. 2007; Row et al. 2007; Solomons et al. 2011). Deubiquitination of internalized tyrosine kinase membrane receptors (RTKs) at the endosomal membranes precedes

their incorporation into MVB vesicles and both AMSH and UBPY have been specifically implicated in the deubiquitination and the regulation of the RTK Epidermal Growth Factor Receptor (EGFR) (Agromayor and Martin-Serrano 2006; Bowers et al. 2006; Clague and Urbe 2006; Mizuno et al. 2006; Row et al. 2006; Alwan and van Leeuwen 2007; Ma et al. 2007; Ali et al. 2013; Savio et al. 2016).

How ESCRT-III activation is achieved to induce polymerization on membranes *in vivo* is however still poorly understood. In the case of the yeast protein Snf7, the N-terminal core domain and the C-terminal α-helix 6 are implicated in this regulation through two different pathways, the ESCRT-I-ESCRT-II-Vps20 pathway and the ESCRT-0-Bro1 pathway (Tang et al. 2015; Tang et al. 2016). In the case of human CHMP4C and CHMP1A, a regulation by phosphorylation has been reported (Maemoto et al. 2013; Caballe et al. 2015).

3. Short Summary of the Results

In the present study, we report for the first time the dynamic ubiquitination of the ESCRT-III protein CHMP1B and its modulation by the deubiquitinating enzyme UBPY. We show that CHMP1B is regulated by mono-ubiquitination in its helical core (K87 and/or K90 residues). Moreover, cell stimulation by EGF, at doses which activates endocytosis of the EGFR, induces a transient enrichment of mono-ubiquitinated dimers of CHMP1B on membranes. Remarkably, solely CHMP1B monomers and dimers are ubiquitinated but not the other oligomeric forms of CHMP1B observed in our study. Additionally, we observed that UBPY interacts with CHMP1B at the level of the late endosomes, catalyzes the deubiquitination of this ESCRT-III protein and favors its oligomerization. Supporting the physiological role of the ubiquitination of CHMP1B, we show that mutation of ubiquitinated lysine residues in the CHMP1B protein prevents the rescue of the EGFR trafficking defects that are observed in *Chmp1B* silenced cells. Using the *Drosophila* model system, we also showed that the wing developmental phenotype induced by *Chmp1* knockdown is rescued by wild type human CHMP1B, but not, or only partially, by the mutated form of human CHMP1B deficient for ubiquitination.

Overall, our findings establish a new ubiquitin dependent mechanism that controls CHMP1B protein activation and polymerization. We propose that CHMP1B ubiquitination serves as a check-point for spatial and temporal control of the ESCRT-III CHMP1B activation at the endosomal membranes.

4. Results

Main figures are indicated as Fig. R

Supplementary figures are indicated as Fig. S

Figures in Annexes are indicated as Fig. A.

4.1 Interaction of CHMP1B with UBPY Occurs via α-helices 4, 5, and 6 of CHMP1B

CHMP1B has been shown to interact with the MIT domain of the ubiquitin protease UBPY in both co-immunoprecipitation and yeast two-hybrid experiments (Row et al. 2007; Agromayor et al. 2009). In order to confirm and further map the domains of CHMP1B implicated in the interaction with UBPY, GFP-tagged constructs of full length or isolated helices of CHMP1B (Fig. R1A) were expressed in HEK293T cells together with a Flag-tagged UBPY and tested for their ability to co-immunoprecipitate with Flag-UBPY. Full-length CHMP1B and the α-helices 4, 5 and 6 were found to interact with Flag-UBPY (Fig. R1B). Interaction was maintained with a catalytically-inactive version of the enzyme ($UBPY^{C748A}$) (Fig. R1C, two upper panels). These results confirm the CHMP1B-UBPY association reported by Row et al (2007) and locate the interaction region between CHMP1B and UBPY to residues 105 to 199 of CHMP1B. This region thus extends beyond the previously described MIM domain as also described for the interaction between CHMP1B and Spastin that involves the residues 148-199 of CHMP1B (Yang et al. 2008).

Strikingly, we observed that co-expression of the two proteins systematically resulted in a higher amount of Flag-UBPY in whole cell lysates (WCL). Indeed Flag-UBPY, which was almost undetectable became strongly stabilized by co-expression of GFP-CHMP1B (Fig. R1C). Then, HeLa cells were transfected with GFP-CHMP1B and transfected GFP-CHMP1B was detected by direct GFP fluorescence while endogenous UBPY was detected by immunostaining. Confocal imaging of transfected cells revealed nuclear and cytoplasmic aggregates of CHMP1B and strong co-localization with UBPY in the cytoplasmic compartment (Fig. S1A). In addition, cells transfected with GFP-CHMP1B displayed higher signals of endogenous UBPY (Fig. S1A, arrows) when compared with neighboring non-transfected cells (Fig. S1A, arrowheads).

Reciprocally, the amount of CHMP1B (both endogenous and expressed GFP-CHMP1B) was increased in the presence of Flag-UBPY or Flag-$UBPY^{C748A}$ in

cell lysates (Fig. R1C). These observation are consistent with the two proteins being partners, as stabilization of the CHMP1B-UBPY complex could very well alter the half-life of each partner.

4.2 Interaction of CHMP1B with UBPY Occurs at Late Endosomes

In order to determine the subcellular localization of the UBPY-CHMP1B complex, we designed a Venus complementation assay in which UBPY was fused to the N-ter of Venus (VN-HA tag) and CHMP1B to the C-ter of Venus (VC-Myc tag) (Fig. R1D). In this assay, a cytoplasmic punctuated signal was clearly observed upon co-expression of VN-UBPY and VC-CHMP1B indicating the complementation of the Venus protein and thereby a direct interaction between the two partners *in vivo*. Co-staining of cells with a set of endosomal markers revealed a strong co-localization of the Venus signal with Lamp-1, a marker of late endosomes/lysosomes, but not with earlier endosomal markers like EEA1 (Fig. R1G, H). Therefore, the two partners mainly interact on late endosomal compartments, a result in agreement with the implication of CHMP1B in MVB biogenesis and receptor sorting (Spitzer et al. 2009; Stuffers et al. 2009). Consistent with the above results, truncation of C-ter helices of CHMP1B led to a decrease (Δα6, Δα5-6) or a fully compromised (Δα4-5-6) signal generation (Fig. R1E, F), confirming that binding of CHMP1B to UBPY occurs through α-helices 4, 5, 6 of the CHMP protein.

4.3 CHMP1B is Ubiquitinated Within its N-terminal Core

We next investigated whether CHMP1B exists in ubiquitinated forms in living cells. The GFP-CHMP1B protein was expressed in HEK293T cells together with HA-ubiquitin (HA-Ub) and immuno-precipitated from whole cell lysate and the presence of ubiquitinated forms of CHMP1B was then analyzed by western blot using anti-HA antibodies. To specifically address the ubiquitination status of the protein CHMP1B and not that of putative partners potentially present in the immunoprecipitated product, highly stringent conditions were used for immunoprecipitation (see Materials and Methods). While the anti-GFP antibody revealed a single band around 55 kDa, i.e, at the molecular weight (*mw*) predicted for GFP-CHMP1B, anti-HA antibodies indicated the presence of a major ubiquitin-linked GFP-CHMP1B product around 70 kDa, likely to represent a mono-ubiquitinated form of the protein, as well as higher *mw* species that may corresponds to multi- or poly- ubiquitinated CHMP1B (Fig. R2A, "WT").

In conditions of overexpression, some cytosolic proteins are modified by addition of K48-ubiquitin chains (UbK48) and targeted for degradation via the proteasome. Using antibodies specifically directed against UbK48, we showed that GFP-CHMP1B does not contain UbK48 (Fig. S1B). Furthermore, inhibiting the proteasomal activity did not influence in the amounts of ubiquitinated GFP-CHMP1B (Fig. S1C).

In preliminary experiments performed on truncated forms of CHMP1B, we observed that GFP-CHMP1B α2 [aa39 to aa92] (Fig. R1A) was preferentially ubiquitinated (not shown). This construct, which includes the end of α1, the entire α2 and the linker region between α2 and α3, contains four lysine residues: K42, K59, K87 and K90 (Fig. R1A). A GFP-CHMP1B mutant in which these four lysine residues were substituted to arginine (CHMP1B-4K>R) presented up to 60% reduction of ubiquitin-linked forms compared to the wild-type CHMP1B (Fig. R2A, A'). We also observed a slight reduction of ubiquitin-linked forms of CHMP1B carrying a single K>R substitution in the case of either the K87>R or the K90>R but not in the case of the K42>R or the K59>R mutations (Fig. R1A, middle and right panels).

These results indicate that CHMP1B is ubiquitinated in human cells and that ubiquitin linkage mostly occurs at the lysine residues K87 or K90 (or both). Remarkably, these lysine residues are located in the flexible linker between α2 and α 3 which adopt a helical rigid structure in the open conformation.

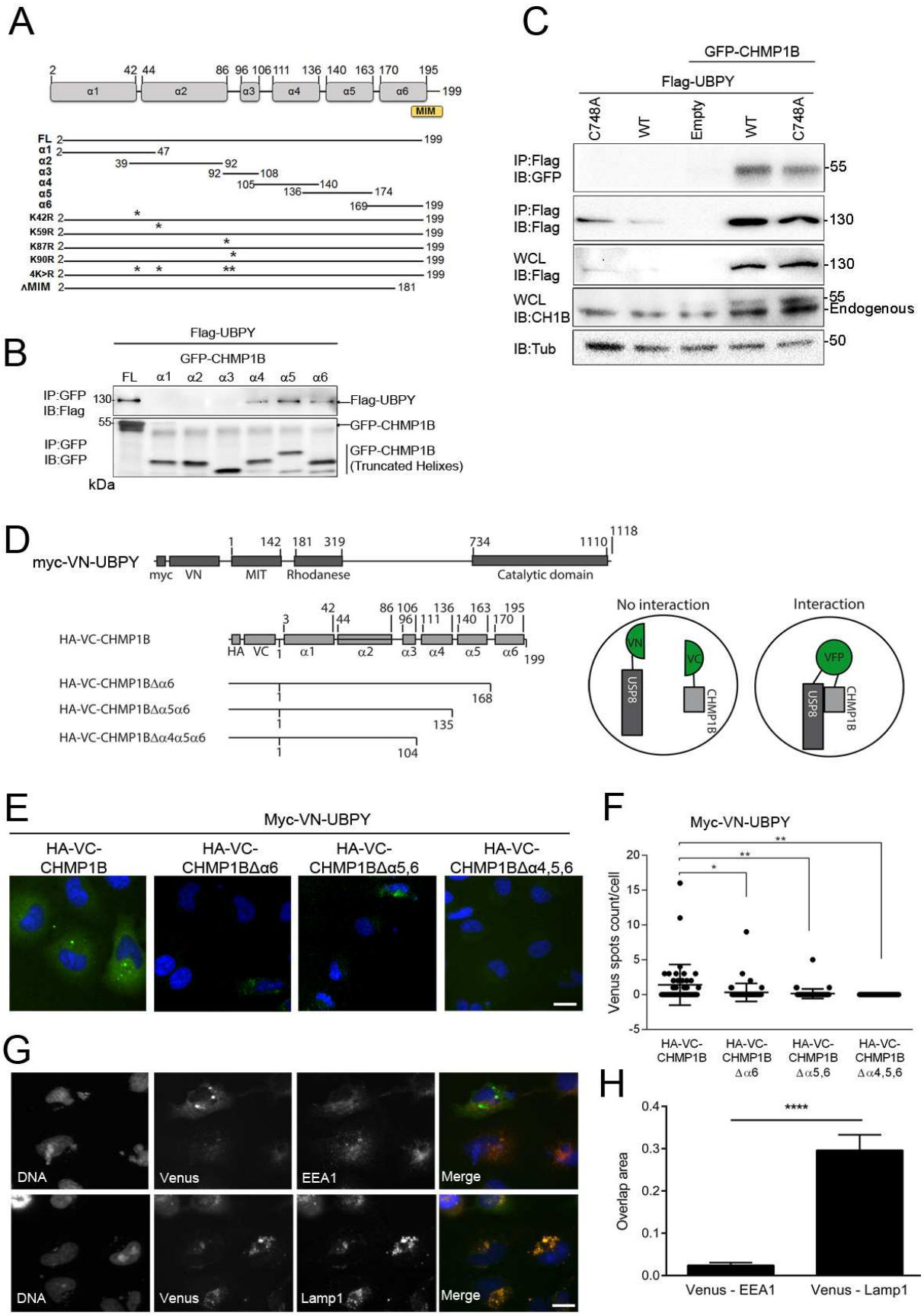

A
MIM
FL
α1
α2
α3
α4
α5
α6
K42R
K59R
K87R
K90R
4K>R
ΔMIM
B
Flag-UBPY
GFP-CHMP1B
FL α1 α2 α3 α4 α5 α6
IP:GFP IB:Flag
IP:GFP IB:GFP
Flag-UBPY
GFP-CHMP1B
GFP-CHMP1B (Truncated Helixes)
kDa
C
GFP-CHMP1B
Flag-UBPY
C748A WT Empty WT C748A
IP:Flag IB:GFP
IP:Flag IB:Flag
WCL IB:Flag
WCL IB:CH1B
IB:Tub
Endogenous
D
myc-VN-UBPY
myc VN MIT Rhodanese Catalytic domain
HA-VC-CHMP1B
HA-VC-CHMP1BΔα6
HA-VC-CHMP1BΔα5α6
HA-VC-CHMP1BΔα4α5α6
No interaction
Interaction
VN
VC
VFP
USP8
CHMP1B
E
Myc-VN-UBPY
HA-VC-CHMP1B
HA-VC-CHMP1BΔα6
HA-VC-CHMP1BΔα5,6
HA-VC-CHMP1BΔα4,5,6
F
Myc-VN-UBPY
Venus spots count/cell
HA-VC-CHMP1B
HA-VC-CHMP1B Δα6
HA-VC-CHMP1B Δα5,6
HA-VC-CHMP1B Δα4,5,6
G
DNA
Venus
EEA1
Merge
Lamp1
H
Overlap area
Venus - EEA1
Venus - Lamp1

Figure R1: UBPY interacts with helices α4, α5 and α6 of CHMP1B

A: Representation of CHMP1B with six predicted α-helices (α1- α6) and cDNA constructs used. The numbers indicate positions of amino acid residues. (*) indicate the lysine residues in positions 42, 59, 87 and 90.

B, C: HEK293T cells were co-transfected with indicated CHMP1B and UBPY constructs and cleared cell lysates were subjected to immunoprecipitation (IP) with anti-GFP or anti-Flag antibodies. IPs were revealed by western blot (IB) using either anti-GFP or anti-Flag antibodies. In (C), whole cell lysates (WCL) were immunoblotted with anti-Flag, anti-CHMP1 or anti-Tub to reveal protein amounts (two lower panels).

D: Schematic representation of the BiFC assay. UBPY was fused to the N-terminal and CHMP1B proteins to the C-terminal fragments of Venus Fluorescent protein (VFP). VFP fluorescence was observed upon direct interaction between individually non-fluorescent VN and VC fusion proteins co-expressed in HeLa cells.

E: Complex formation between UBPY and CHMP1B proteins requires α4, α5 and α6 helixes in HeLa cells. BiFC complexes by VFP fluorescence (Sorkin et al.). All images were taken under the same magnification. Scale bars: 20 μm.

F: Quantification of UBPY-CHMP1B BiFC VFP spots was performed using HCS Studio software. Vertical axis indicates Venus spots count per HA and Myc positive cell. Scatter dot plots represent one representative experiment out of three. Values are mean ± SD. *$p<0.01$; **$p<0.05$ (Student's *t*-test).

G: HeLa cells co-transfected with myc-VN-UBPY and HA-VC-CHMP1B were fixed, permeabilized and immunostained for the early endosomal marker EEA1 and late endosomal/lysosomal protein LAMP1. Low levels of DNA wer used to avoid big aggregates.

H: Quantification of the co-localization between UBPY-CHMP1B VFP spots and EEA1, or LAMP1 is represented as a fraction of VFP fluorescence overlapping the indicated endosomal markers. Vertical axis indicates overlap area. Histograms represent one representative experiment out of three. Values are mean ± S.E.M. ****$p<0.0001$ (Student's *t*-test).

4.4 The Ubiquitination Status of CHMP1B is Controlled by UBPY

Since UBPY is a main partner of CHMP1B (Row et al. 2007) we tested whether ubiquitinated CHMP1B is a target of the ubiquitin hydrolase activity of UBPY. To this end, HEK293T cells were co-transfected with HA-Ub and GFP-CHMP1B together with silencing or overexpressing constructs of wild-type or mutated UBPY. Silencing of *Ubpy* resulted in a slight increase of the ubiquitinated pool of GFP-CHMP1B (Fig. R2B, B'). In contrast, the over-expression of UBPY but not of the catalytic mutant $UBPY^{C748A}$ caused a strong reduction of the Ub-CHMP1B pool which was even further reduced when expressing the constitutively active form of the enzyme: $UBPY^{S680A}$ (Fig. R2B, B'). Our results thus strongly suggest that UBPY deubiquitinates CHMP1B and that a complete c-terminal domain of CHMP1B is necessary to interact properly with UBPY.

4.5 CHMP1B is Transiently Ubiquitinated Upon Cell Stimulation by EGF

Given the role of the ESCRT machinery in the downregulation of a vast array of receptors, including EGFR and the pro-inflammatory cytokine receptors, TNFR and IL1R, in response to their ligands, we tested the effect of EGF, TNFα and IL1β on CHMP1B ubiquitination when added at doses known to induce the internalization of their respective receptors (Brissoni et al. 2006; Sigismund et al. 2013; Windheim and Hansen 2014; Mamińska et al. 2016). In cells co-expressing GFP-CHMP1B and HA-Ub, a clear and transient elevation of the amount of ubiquitinated GFP-CHMP1B was observed at 5 min of stimulation with EGF (Fig. R2C). This timing coincides with the onset of the interaction between the EGFR and the ESCRT proteins (Ali et al. 2013). The amount of ubiquitinated GFP-CHMP1B then diminished to reach its basal level at 10 min of stimulation (Fig. R2C). In the case of TNFα or IL1β treated cells, a similar transient accumulation of ubiquitinated GFP-CHMP1B was observed at 20 and 10 min of stimulation, respectively (Fig. S2A, B). In these two cases, a second pic of CHMP1B ubiquitinated pool was detected at 20 and 60 min respectively. Several rounds of ubiquitination-deubiquitination of target proteins during activated receptor trafficking have been previously proposed (Madshus and Stang 2009).

These experiments show that ubiquitination of CHMP1B is regulated in response to EGF or cytokines stimulation at specific kinetics depending on the endocytic signal.

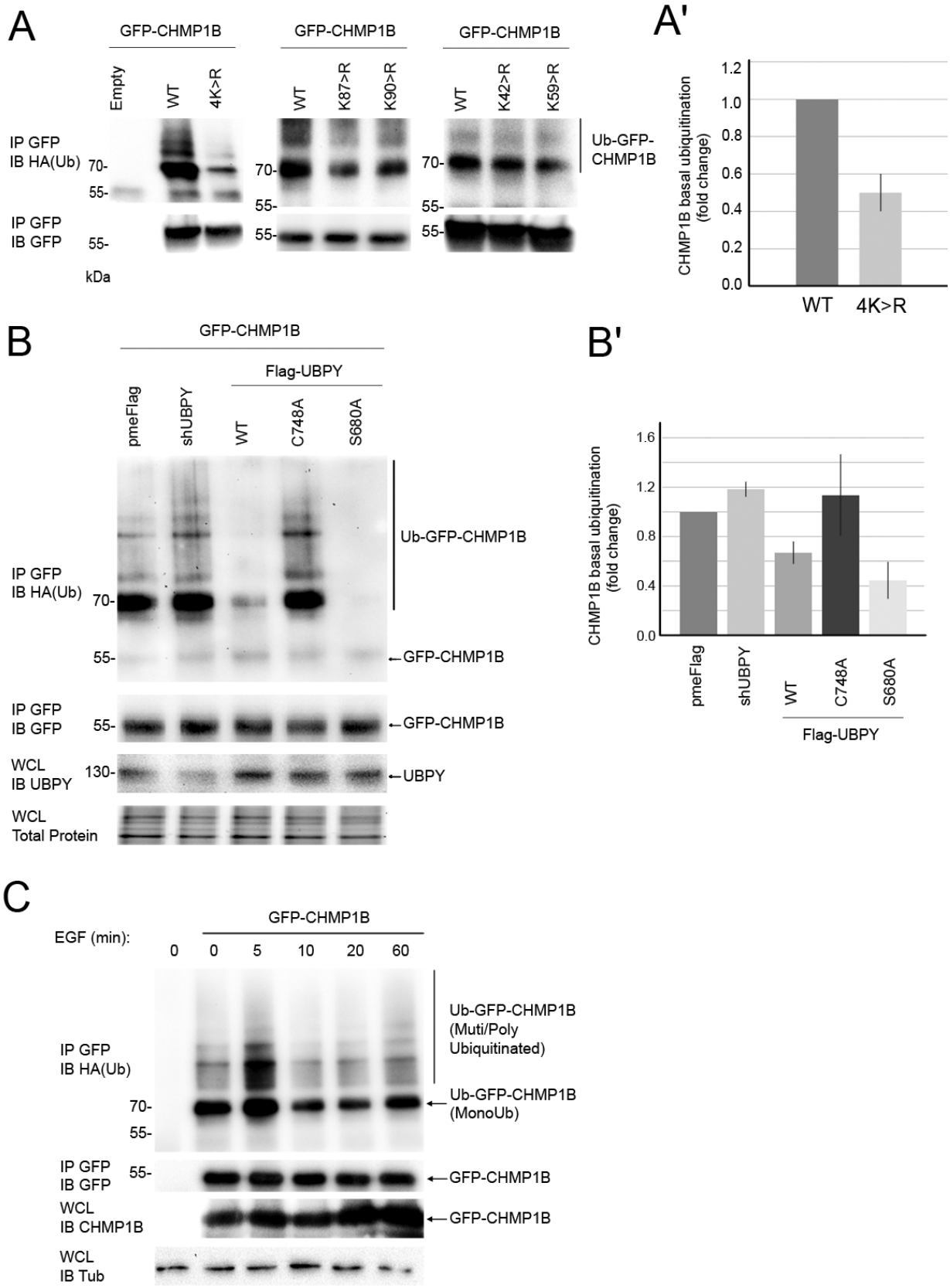

A
GFP-CHMP1B
Empty
WT
4K>R
K87>R
K90>R
K42>R
K59>R
IP GFP
IB HA(Ub)
IP GFP
IB GFP
70-
55-
kDa
Ub-GFP-CHMP1B
A'
CHMP1B basal ubiquitination (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
WT
4K>R
B
Flag-UBPY
pmeFlag
shUBPY
C748A
S680A
GFP-CHMP1B
WCL
IB UBPY
130-
WCL
Total Protein
B'
1.6
1.2
0.8
0.4
0.0
C
EGF (min):
0
5
10
20
60
Ub-GFP-CHMP1B (Muti/Poly Ubiquitinated)
Ub-GFP-CHMP1B (MonoUb)
WCL
IB CHMP1B
WCL
IB Tub

Figure R2: CHMP1B ubiquitination pattern and dynamic

A: Full-length or mutated (87K>R; 90K>R; 59K>R, 42K>R or 4K>R) GFP-CHMP1B constructs were transfected into HEK293T jointly with HA-ubiquitin (HA-Ub). Immunoprecipitations (IPs) were carried out with anti-GFP antibodies after strong denaturation of the lysate and proteins were analyzed by western blot (IB) using either anti-HA or anti-GFP antibodies.

B: Ubiquitination of CHMP1B is regulated by UBPY catalytic activity. Similar experiments as in A. HA-Ub and GFP-CHMP1B full-length construct were transfected into HEK293T together with sh*Ubpy* or Flag-UBPY, Flag-UBPY^{C748A} or UBPY^{S680A} constructs.

A', B': Quantification by densitometry of blots in A or B, respectively. Normalized signals are expressed as a fold-change over CHMP1B full-length basal ubiquitination levels. Histograms represent the mean of two independent experiments. Error bars indicate standard deviation.

C: HEK293T cells were transfected for 48h with GFP-CHMP1B and HA-Ub constructs. Cells were subjected to serum starvation for 6 h prior to stimulation by EGF at 100 ng/ml for the indicated time prior to a strong denaturing lysis. Cleared cell lysates were subjected to IP with anti-GFP antibodies. IPs were revealed by IB using either anti-GFP or anti-HA antibodies.

4.6 Endogenous CHMP1B Forms Resistant Oligomers

To analyze the behavior of the endogenous protein, we used a polyclonal antibody raised against the epitope formed by the amino acids 35 to 84 of human CHMP1B. Preliminary characterization of this antibody indicated that in western blot experiments, it recognized SDS-PAGE separated and denatured GST-CHMP1B (Fig. S3). Based on the crystal structure of CHMP1B (McCullough et al. 2015), this epitope is likely masked by the regulatory domain in the closed conformation of CHMP1B, which would prevent its recognition by the antibody when experiments are conducted in non-denaturing conditions (Fig S3A).

The analysis of the whole cell lysate from HEK293T or HeLa cells with the anti-CHMP1B antibody revealed three major bands at 25-28 kDa, 55 KDa and 200 kDa that were massively diminished upon silencing of *chmp1B* using two

independent shRNA (Fig. S3B). These results confirm the specificity of the antibody towards CHMP1B and indicate that endogenous CHMP1B is present in distinct species possibly corresponding to monomers, dimers and higher order SDS-resistant complexes.

Extra heat denaturation of the samples resulted in a loss of the 200 kDa band and an increase of the 55 kDa band, supporting the hypothesis that the high *mw* species correspond to an SDS-resistant oligomeric form of CHMP1B (Fig. S3C). These sub-complexes were then separated on a sucrose step gradient after cell lysis in the presence of 1% NP40. In this case, CHMP1B oligomers were found in the 30% sucrose fraction and in lower amount in the 20% fraction while dimers accumulated in lighter fractions. Monomeric band was not detected in any of the three replicates of the experiment (Fig. R3A). The lysates were ten times concentrated than in previous experiments and it is possible that in this high protein density conditions, the monomers disappears because they associate to form higher order complexes as observed *in vitro* (McCullough et al. 2015).

Ist1 can form polymeric structures with CHMP1B either *in vitro* or when co-overexpressed within cells (McCullough et al. 2015). This protein was detected only in the lower sucrose gradient fractions (0%, 10%, 20% fractions) in two forms of 32kDa and 40kDa that may correspond to monomers with or without posttranslational modifications or different charges or conformations. The 40kDa Ist1 band appear in 20% sucrose fraction and in small amounts in the 30%, both of which contain CHMP1B oligomers. (Fig. R3A). Thus, the 40kDa band may correspond to a form of Ist1 that associates with CHMP1B in higher order complexes.

In contrast to the endogenous protein, the GFP-tagged CHMP1B was detected only as monomers using either the anti-GFP or the CHMP1B antibody (Fig. R2). Actually, the presence of the N-terminal GFP tag may impede protein assembly and generation of dimers and oligomers. GFP-CHMPs proteins have been reported to act as a dominant negatives (Martin-Serrano et al. 2003; Strack et al. 2003; Zamborlini et al. 2006) putatively because of the defect in their polymerization capacity.

Taken together, our results indicate that CHMP1B forms SDS-resistant dimers and oligomers alone or in complex with some other ESCRT proteins.

4.7 Monomers and Dimers of Endogenous CHMP1B are Ubiquitinated

We next analyzed if endogenous CHMP1B is also ubiquitinated. To this end, ubiquitinated proteins from HEK293T cells were immunoprecipitated using the

anti-ubiquitin FK2 antibody and further analyzed by Western blot using the previously-characterized anti-CHMP1B antibody.

In the pool of ubiquitinated proteins, immunoblotting revealed two differentially abundant CHMP1B-positive bands corresponding in size to a mono-ubiquitinated CHMP1B monomer (35 kDa, low amount) and to a dimer of mono-ubiquitinated CHMP1B (70 kDa, high amount) (Fig. R3B). Since ubiquitinated CHMP1B was not detected in the full cell lysate or only as very faint bands, it must represent a minor part of total CHMP1B within the cell (Fig. R3B, WCL). In contrast to monomers and dimers, high *mw* species of CHMP1B were not found in the ubiquitinated fraction (Fig. R3B).

In parallel experiments, endogenous CHMP1B was immunoprecipitated from HEK293T cell extracts and CHMP1B was detected by immunoblotting with anti-CHMP1B. While monomers (25 kDa), dimers (55 kDa) and oligomers (200 kDa) could be detected in the whole cell lysate as previously described (Fig. R3C, WCL), only the oligomers and the ubiquitinated monomers (35 kDa) and dimers (70 kDa), but not the non-ubiquitinated monomers and dimers of CHMP1B, were found in the CHMP1B fraction immunoprecipitated from non-denatured cell extracts (Fig. R3C, IP: CHMP1B). The oligomers are also efficiently immunoprecipitated by the anti-CHMP1B antibody. This results indicate that in the ubiquitinated forms of CHMP1B as well as in the oligomers, the recognized epitope is exposed for antibody binding.

Performing the same kind of experiment, oligomeric forms of CHMP1B were repeatedly found in larger amounts in Flag-UBPY overexpressing cells compared to control cells (Fig. R3D).

Taken together, our results demonstrate the existence of endogenous ubiquitinated CHMP1B monomers and dimers while CHMP1B oligomers are not ubiquitinated. Furthermore, they suggest that the hydrolysis of ubiquitin by UBPY favors CHMP1B oligomerization.

We then tested the effect of EGF stimulation on the ubiquitination profile of endogenous monomers and dimers of CHMP1B. HEK293T cells were treated with 100 ng/ml EGF and ubiquitinated proteins were immunoprecipitated using the FK2 antibody at different times of the kinetics. As previously observed with the GFP-CHMP1B protein, a transient although modest increase of the amount of Ub-CHMP1B, mostly visible on the dimer forms, was observed at 5 min post-stimulation with EGF. Return to basal levels followed at 15 to 30 min of EGF treatment (Fig. R3E). Fractionation of the post-nuclear supernatant in cytosolic *versus* membrane fractions prior immunoprecipitation revealed that the pool of CHMP1B dimers ubiquitinated in response to EGF was strongly enriched in the

membrane fraction (Fig. R3E, pellet). Nuclear versus cytoplasmic fractions were analyzed in the same way and the results confirmed that the accumulation of ubiquitinated dimers of CHMP1B does not correspond to the nuclear fraction of the protein (Fig. S4).

From these results, we conclude that endogenous CHMP1B is rapidly ubiquitinated upon EGF stimulation and transiently accumulate on membranes as ubiquitinated dimers.

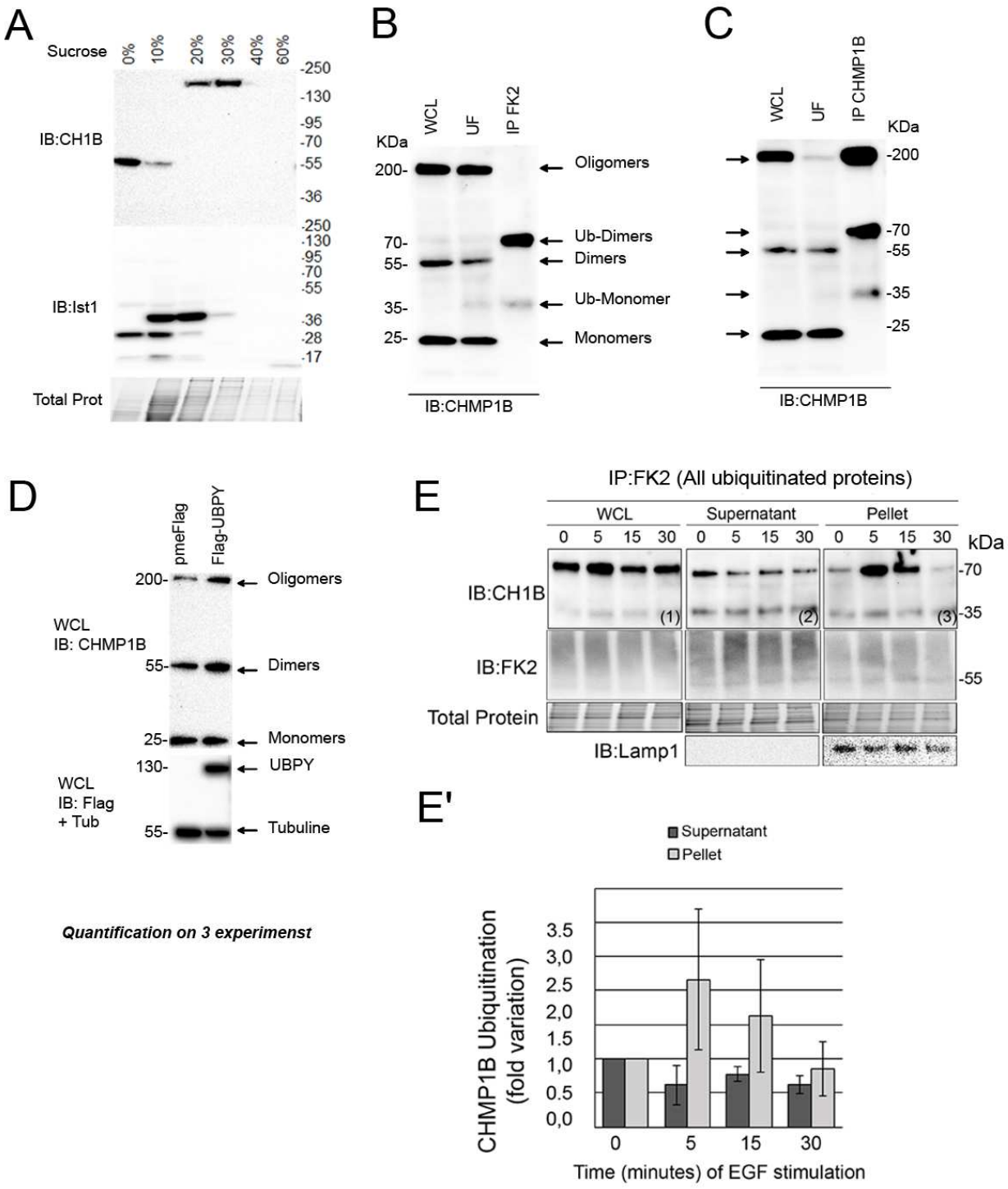


Figure R3: CHMP1B oligomers are not ubiquitinated and Ub-CHMP1B transiently accumulates in the membranes after EGF stimulation

A: Non-treated HEK293T cells were lysed in RIPA buffer and the protein content was separated in a sucrose gradient. The proteins content of different fractions were separated by SDS-PAGE and revealed by IB using anti-CHMP1B or anti-Ist1

B: Non-treated HEK293T cells were lysed in RIPA buffer and all ubiquitinated proteins (IP-FK2) were immunoprecipitated with the indicated antibody. The whole cell lysate (WCL), the unbound fraction (UF) and the IP-FK2 were analyzed by IB against CHMP1B.

C: Non-treated HEK293T cells were lysed in RIPA buffer and endogenous CHMP1B (IP-CHMP1B) was immunoprecipitated with the indicated antibody. The whole cell lysate (WCL), the unbound fraction (UF) and the IP-CHMP1B (right) were analyzed by IB against CHMP1B.

D: Flag-UBPY constructs was transfected into HEK293T as indicated. WCL were analyzed by western blot using either anti-CHMP1B or anti-Flag and anti-tubulin antibodies.

E: HEK293T cells were subjected to EGF 100ng/ml stimulation during the indicated times. Cell fractions were prepared and ubiquitinated proteins were immunoprecipitated with anti-FK2 under non-limiting antibody conditions, from either the WCL (1) or the supernatant (2) or the pellet (Sweeney and Carraway). IPs analyzed by SDS-PAGE and revealed by IBs using anti-CHMP1B.

E': Quantification by densitometry of blots in E. Normalized signals are expressed as a fold-change over CHMP1B basal ubiquitination levels at time 0 minutes (in the absence of EGF). Histograms represent the mean of two independent experiments. Error bars indicate standard deviation.

3.8 Ubiquitination of CHMP1B is Required for EGFR Trafficking

In order to investigate the physiological relevance of CHMP1B ubiquitination, we analyzed the behavior of EGFR in 3'UTR-*chmp1B*-silenced cells expressing the shRNA resistant versions of either wild-type or 4K>R mutant CHMP1B. Expression levels of CHMP1B proteins were verified by western blot (Fig. S5). The amount of EGFR at the plasma membrane was followed by immunostaining on non-permeabilized cells at different time point post-EGF stimulation. As shown in Fig. R4A treatment of HeLa cells with 100 ng/ml EGF induced the progressive removal of EGFR from the plasma membrane in the ensuing 30 min and a complete loss of staining at 60 min post-stimulation. We observed that silencing of c*hmp1B* altered this kinetics as EGFR staining at the plasma membrane was reduced but still

present at 60 min. The internalization process *per se* was not significantly affected, as permeabilized cells displayed similar accumulation of internalized EGFR in both control and *chmp1b*-silenced cells at 30 min post-EGF addition. (Fig. R4B) Thus, persistence of EGFR at the plasma membrane in *chmp1b* silenced cells would be consistent with a defect in EGFR sorting, in which a decreased lysosomal degradation would be coupled with an enhanced recycling rate of EGFR at the plasma membrane. Interestingly, whatever the mechanism, the expression of CHMP1B-WT in *chmp1B*-silenced cells, but not of CHMP1B-4K>R, was able to rescue the kinetics of EGFR disappearance at the membrane (Fig. R4A), clearly supporting a physiological role for CHMP1B ubiquitination in EGFR trafficking.

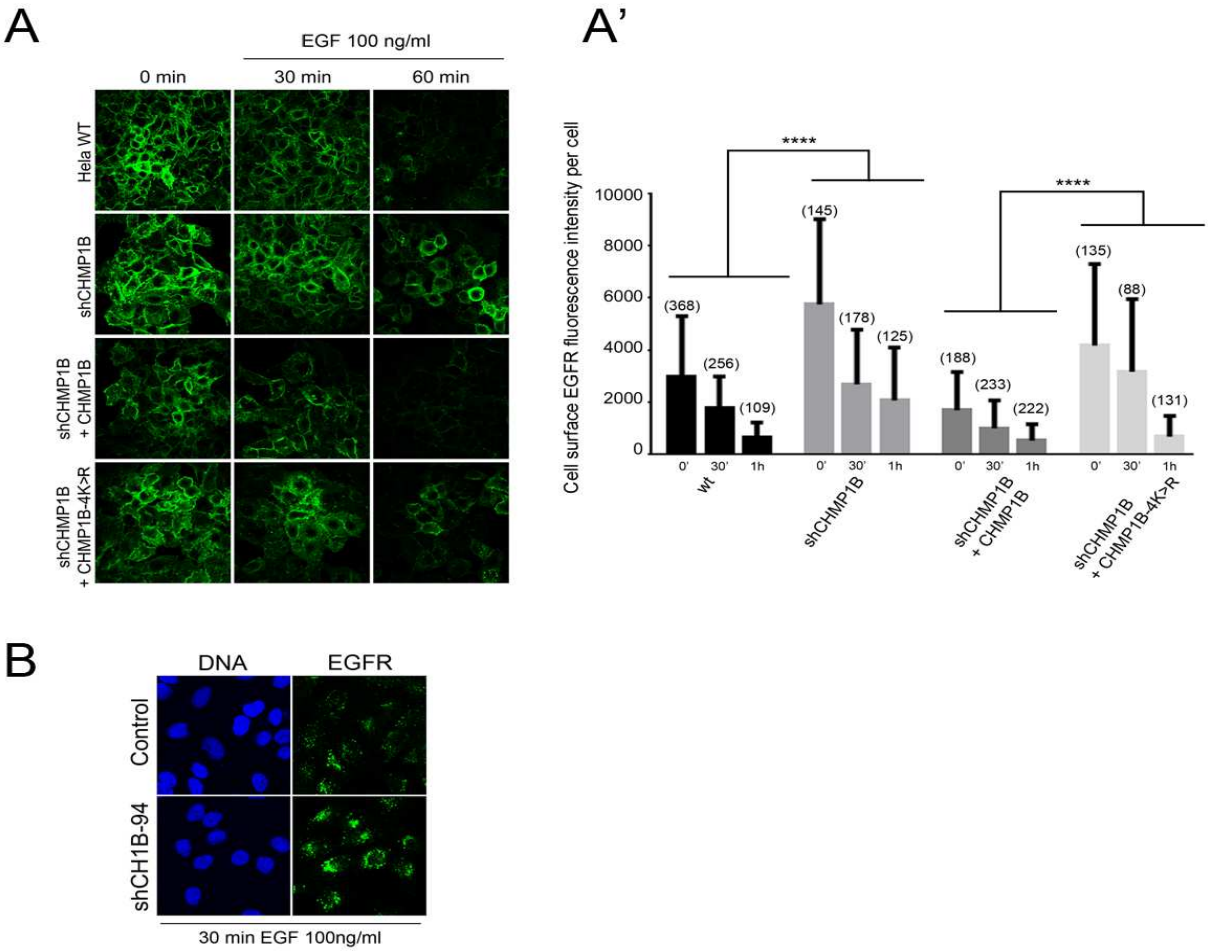


Figure R4: Ubiquitination of CHMP1B is required for EGFR trafficking.
A: Confocal images of HeLa cells or *Chmp1B*-silenced HeLa cells (sh94) transfected with HA-empty, HA-CHMP1B or HA-CHMP1B-4K>R. Cells were subjected to serum starvation prior to stimulation by EGF and fixed at the indicated time points.

Immunofluorescence staining of non-permeabilized cells was performed to stain specifically plasma membrane EGFR.
A': Quantification of the intensity of fluorescence per cell was performed using Cell Profiler Software. Vertical axe indicate fluorescence intensity units per cell. Histograms represent the mean intensity measured from individual cells. Error bars indicate standard deviation. The numbers between parentheses indicate the number of cells measured for each experimental condition. Statistical significance was determined using two-way ANOVA (****p<0.0001).
B: Confocal images of HeLa cells or *Chmp1B*-silenced HeLa cells (sh94). Cells were subjected to serum starvation prior to stimulation by EGF and fixed at 30 min of stimulation. Immunofluorescence staining was performed on permeabilized cells using anti-EGFR.

3.9 Ubiquitination of CHMP1B is required for Drosophila Wing Development

To assess the generality of a CHMP1B regulation by the ubiquitin system, we extended our experiments to the *Drosophila melanogaster* model system. The *Drosophila* genome encodes one orthologue of Hs-UBPY, the protein *DmelUBPY* (syn. USP8) that shares 45% sequence identity with its human counterpart and a unique CHMP1 protein, *DmelCHMP1,* displaying 90% identity with human CHMP1B (Pradhan-Sundd and Verheyen 2015). Three out of the four lysine residues targeted in this work are conserved and the MIM domain is almost identical except for one conservative substitution (Fig. R5A).

Ubiquitous silencing of *Chmp1* or *Ubpy* in *Drosophila* is lethal before the pupal stage (our unpublished observations), so we used transgenic fly lines expressing silencing hairpins under the control of UAS promoter (see annex I.B): UAS-CHMP1-IR {(Dietzl et al. 2007) and/or UAS-UBPY-IR, Horn and Boutros, DKFZ, Heidelberg)} and a specific wing driver Gal4 (MS1096-Gal4) inducing the expression of the UAS constructs in the dorsal wing layer. Consistent with previous studies showing the role of these two genes in wing development, (Valentine et al. 2014; Pradhan-Sundd and Verheyen 2015) silencing either *Chmp1* or *Ubpy* resulted in a similar curved and growth defective wing phenotype (Fig. R5B). Co-silencing both genes produced a synergistic phenotype abrogating wing formation (Fig. R5B). As protein depletion is usually incomplete in gene silencing experiments, the observed synergistic defect suggest that both proteins act in common processes leading to wing formation.

During wing development, a complex set of signaling events establishes a row of active Notch pathway cells in the wing larval imaginal disc, at the level of the future wing margin (Irvine and Vogt 1997; Irvine 1999) and see annex II. Using a EGFP reporter gene expressed under Notch Responsive Elements (NRE) and the driver *engrailed (en)*-Gal4 to induce the silencing construct in the posterior part of the imaginal wing disc (see annex I.D), we observed that silencing endogenous *Chmp1* resulted in an enlarged domain of EGFP expression both in the domain of En expression and slightly beyond (Fig. R5C), consistent with a cell-autonomous and non-cell autonomous role of ESCRT proteins in the regulation of developmental signals (Cadigan 2002; Zecca and Struhl 2002a; Zecca and Struhl 2002b; Rives et al. 2006; Vaccari et al. 2009; Aoyama et al. 2013; Matusek et al. 2014; Valentine et al. 2014; Jacomin et al. 2016). This resulted in observable wing defects at the adult stage (Fig. R5C).

Transgenic flies conditionally expressing human transgenes encoding either wild type *Hs-CHMP1B* or *Hs-CHMP1B-4K*>R were then generated to test their ability to rescue the wing phenotype induced by *Dmel-Chmp1* silencing. Remarkably, restriction of the Notch signaling domain in larval imaginal discs was fully restored by expressing wild-type Hs-CHMP1B-WT resulting in normal adult wings. In contrast, expressing Hs-CHMP1B-4K>R mutant resulted in a high proportion of adults presenting wing defects (85%) and a few adults with normal wings (15%) (Fig. R5C). Similar experiments were performed using the MS1096 driver with similar results (Fig. S6). Partial rescue by the ubiquitination defective Hs-CHMP1B-4K>R mutant indicates that modification of CHMP1B by ubiquitin linkage contributes to the protein function in *Drosophila* thus providing evidence for the evolutionary conservation of an ubiquitination-based regulation of *Hs*CHMP1B and *Dme*lCHMP1 proteins.

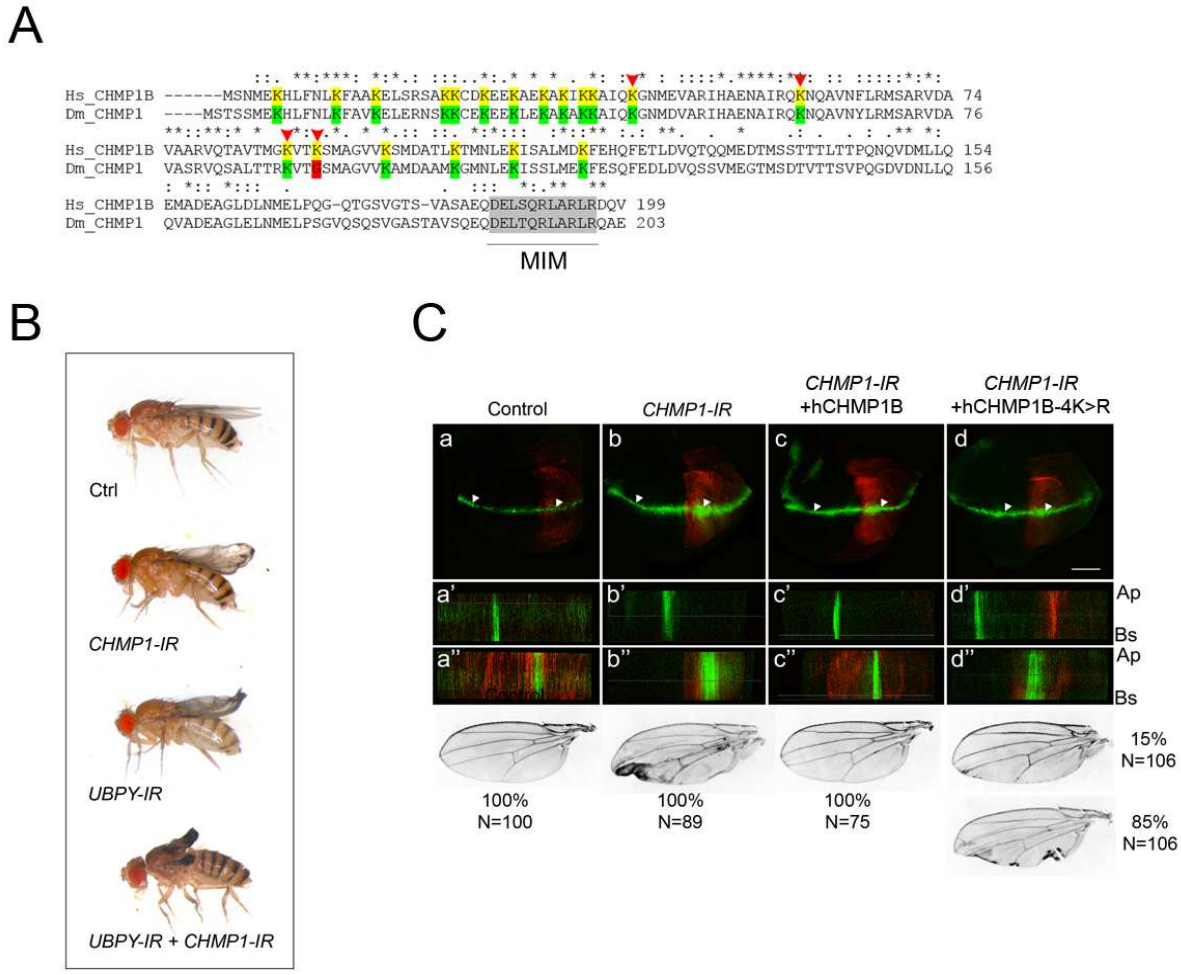


Figure R5: The ubiquitin deficient form of CHMP1B is not functional during *Drosophila* wing development.

A: Protein sequence alignment of *Drosophila melanogaster* CHMP1 versus its *Homo sapiens* horthologous CHMP1B. In green are marked the lysine residue conserved between both species and in red, the one which is not. The 4 lysine residues substituted by arginine used in this article are marked with red arrow-heads. The MIM (MIT interacting domain) is marked in grey.

B: Pictures of 3 to 5 day-old female flies raised at 18°C (panel 1) or 25°C (panel 2) expressing the indicated transgenes under the control of the wing MS1096-Gal4 driver. The balance of UAS sequences has been respected. Genotypes are as follows in w^{1118} background. Panel 1 (synergy): **Ctrl:** MS1096/+. ***CHMP1-IR:*** MS1096/+; UAS-lacZ/+; *UAS-dCHMP1(IR)/+*. ***UBPY-IR*:** MS1096/+; *UAS-UBPY-IR/+; UAS-birA*. ***UBPY-IR + CHMP1-IR*:** MS1096/+; *UAS-UBPY-IR/+; UAS-*

dCHMP1-IR/+. . Number of flies examined are N>100 (50% females + 50% males) in all cases. The phenotypes are 100% penetrating in all cases.

C: Confocal images of late L3 larvae wing imaginal discs dissected as described in supplemental material. EGFP is expressed under the control of Notch Responding Elements (NRE). Myr-RFP is express under the control of a UAS sequences. Gal4 is express under the control of engrailed (en) enhancer element. Genotypes are as follows: **Ctrl:** *w^{1118} ; en-Gal4, UAS-Myr-RFP, NRE-EGFP/+*. ***CHMP1-IR:*** *w^{1118}*; *en-Gal4, UAS-Myr-RFP, NRE-EGFP/+*; *UAS-dCHMP1(IR)/+*. **hCHMP1B *+ CHMP1-IR*:** *w^{1118}*; *en-Gal4, UAS-Myr-RFP, NRE-EGFP/UAS*-hCHMP1B; *UAS-dCHMP1(IR)/+*. **hCHMP1B-4K>RR *+ CHMP1-IR*:** *w^{1118}*; *en-Gal4, UAS-Myr-RFP, NRE-EGFP/UAS*-hCHMP1B-4K>RR; *UAS-dCHMP1(IR)/+*. ***a,b,c,d:*** frontal average Z stack of confocal series. **a'** and **a"** show lateral views of the Z stack in **a** at the level of the non-Gal4 expressing area (a') or the Gal4 expressing area (a") indicated by the arrowheads. Same configuration for **b', b", c', c", d', d"**. **Ap**: apical side. **Bs:** basal side. Magnification objective 63x**.** **Scale bar**: 75µm

5. Supplementary Figures

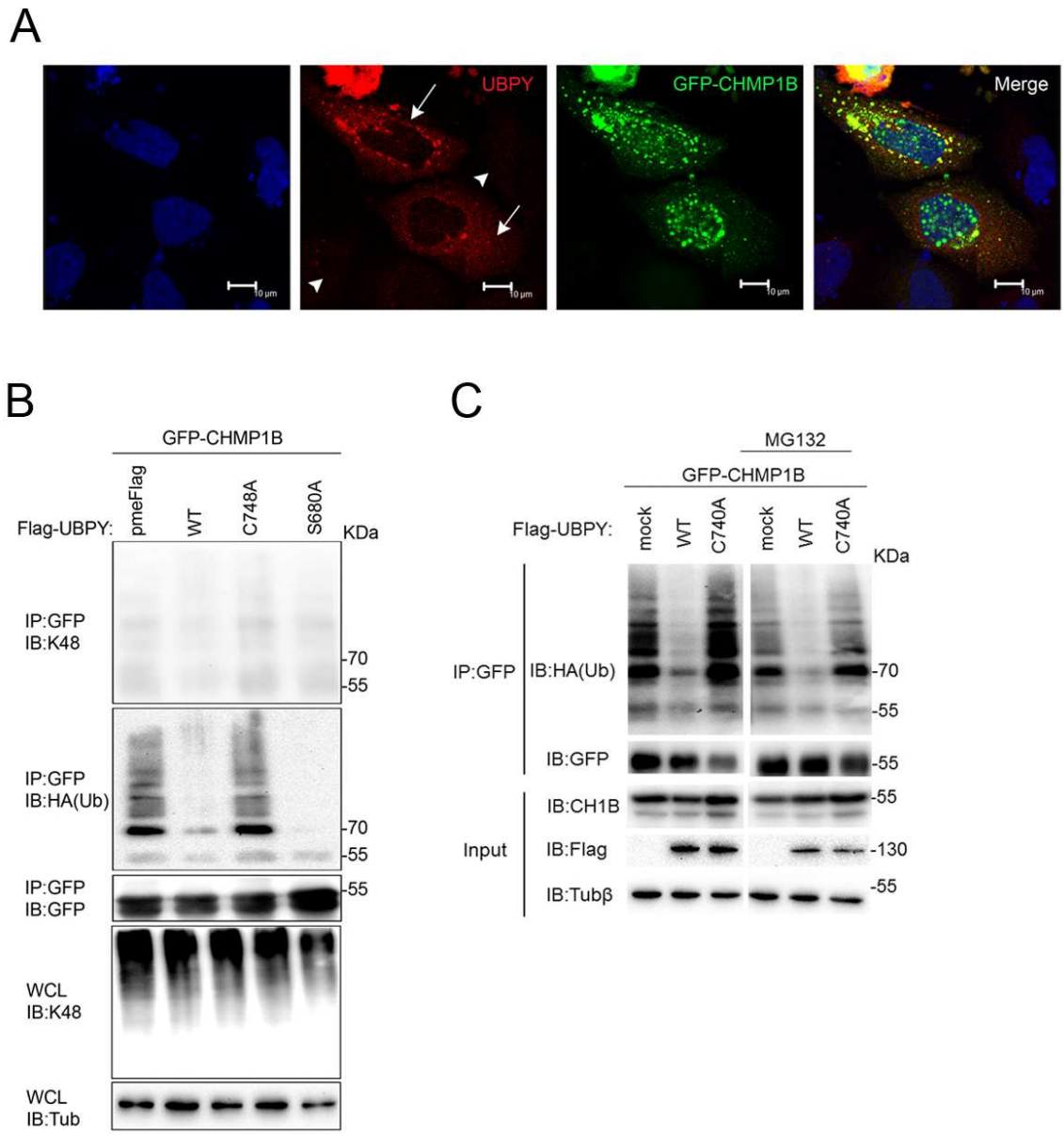


Figure S1: A: Subcellular localization of UBPY and CHMP1B in HeLa cells HeLa cells were transfected with GFP-CHMP1B for 24 h and then fixed, stained with anti-UBPY antibody and mounted for confocal microscopy. The stabilization of endogenous UBPY in cells positive for GFP-CHMP1B (arrows) compare to the neighbors non-transfected cells (arrow heads). Magnification objective 63x. Scale bar: 20 µm. **B:** The same experiments as in Fig. R2B were performed in HEK293T cells and immunoprecipitated GFP-CHMP1B was analyzed by western blot with anti-K48 or anti-HA (Ub) or anti GFP (control) antibodies. Whole cell lysate was analyzed by immunoblot with anti-K48 or anti Tubulin (Tub). **C:** The same

experiments as in Fig. R2B were performed in HEK293T in presence or absence of proteasomal activity blocker MG132. Immunoprecipitated GFP-CHMP1B was analyzed by western blot with anti-GFP or anti-HA (to reveal HA-Ub).

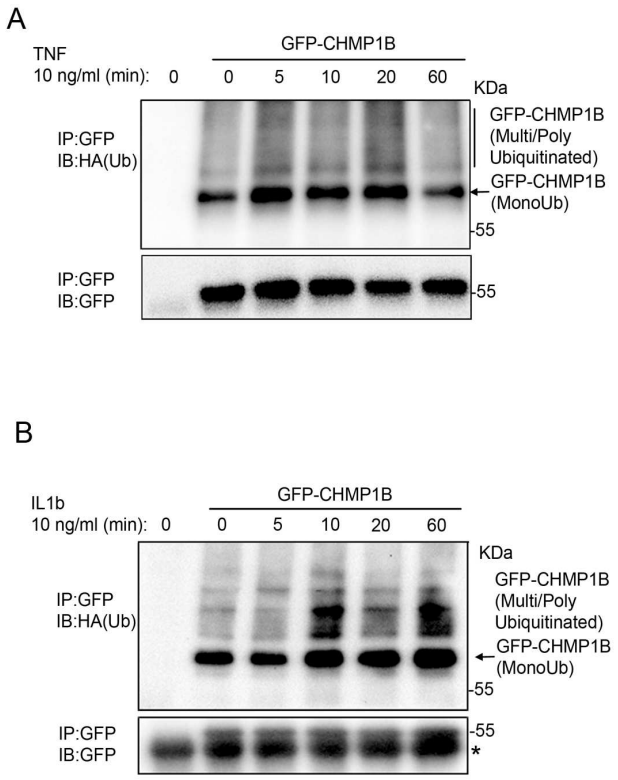


Figure S2: Dynamic CHMP1B ubiquitination induced by cytokines. HEK293T cells transfected with HA-Ub and GFP-CHMP1B were stimulated with 10 ng/ml of TNF (**A**) or 10 ng/ml of IL1β (**B**) 48 hours after transfection. At indicated times, cells were subjected to strong denaturation lysis and GFP-CHMP1B was immunoprecipitated from the cleared lysates using anti GFP antibodies. Immunoprecipitated proteins were analyzed by Western blot using anti-GFP or anti-HA antibodies. *The band below the 55 kDa in (B) IB:GFP corresponds to the IgGs.

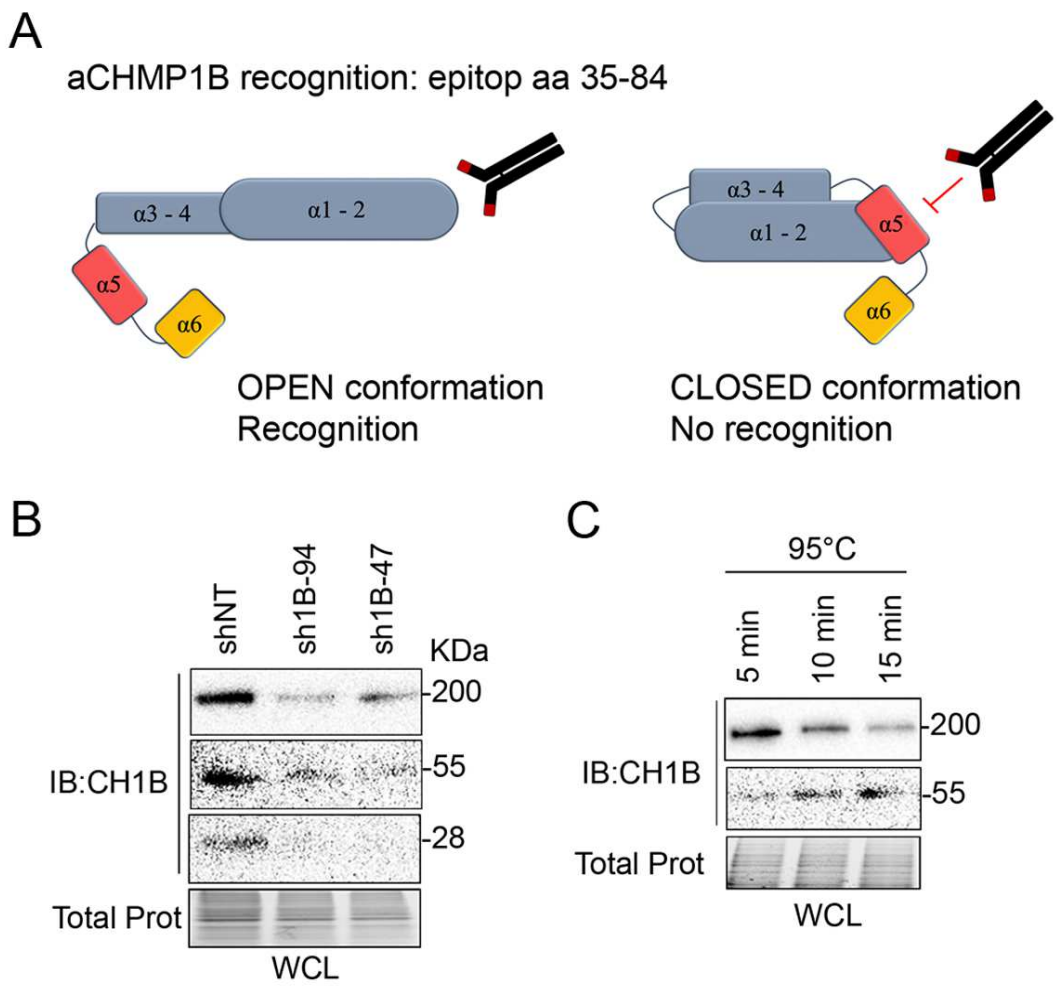


Figure S3: A: Schematic representation of the recognition site of the conformational antibody against CHMP1B. **B:** Analysis of lysates from Hela cells expressing or not a shRNA-CHMP1B against 3'UTR region (sh94) or the CDS (sh47). Proteins were separated by SDS-PAGE and revealed by IB using anti-CHMP1B on cut membranes to improve the efficiency of the antibody. **C** Non-treated HEK293T cell lysates were incubated at 95°C during the indicated times in the presence of Lammeli1x. Proteins were separated by SDS-PAGE and revealed by IB using anti-CHMP1B on cut membranes.

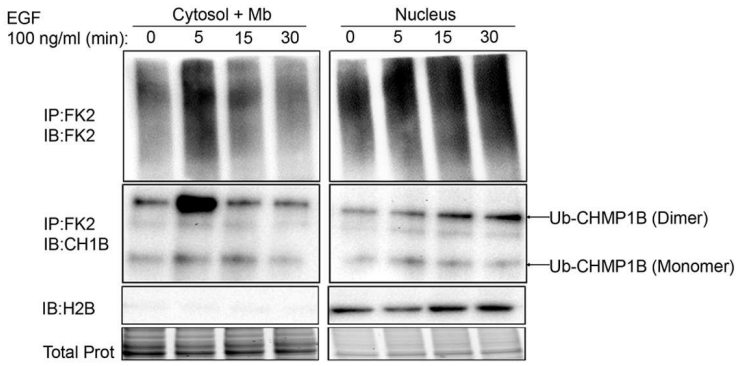


Figure S4: HEK293T were subjected to EGF stimulation and cells lysates were fractionated at different time points in cytosolic and nuclear fractions using NE-PER® Nuclear and Cytosolic Extraction Reagents (Prod # 78835) following the instructions provided by the supplier. Cleared cell fractions were subjected to immunoprecipitation. All ubiquitinated proteins were immunoprecipitated with the FK2 antibody. Immunoprecipitated proteins were analized by western blot analysis using either anti-CHMP1B or anti-FK2 antibodies.

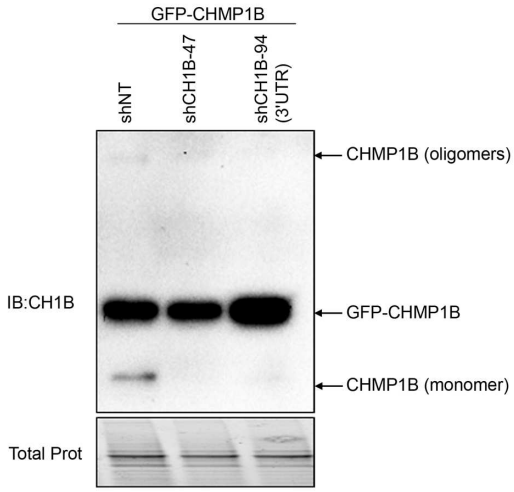


Figure S5: Validation of CHMP1B extinction in shRNA lines. Hela cells were stably transduced with shRNA non-target, shRNA-CHMP1B TRCN0000159294 (targets 3'UTR region) or shRNA-CHMP1B TRCN0000165547 (targets CDS region) from Mission Sigma shRNA library. Cells were transfected with GFP-CHMP1B construct for 48 hours and whole lysates were analyzed by western blot using anti-CHMP1B. Endogenous oligomers and monomers are indicated. Dimer signal is masked by the over-expressed GFP-CHMP1B.

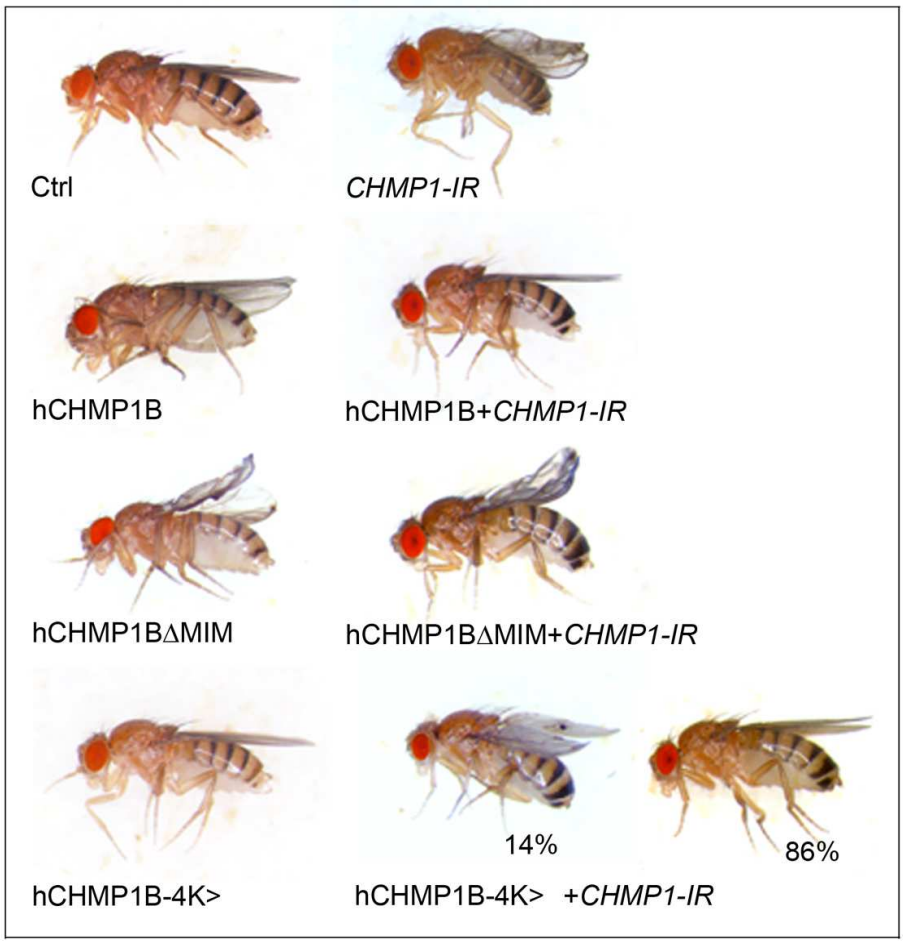


Figure S6: Picture of 3-5 day-old males and female flies raised at 25°C and expressing indicated transgenes under the control of the MS1096Gal4 driver which is expressed mostly in the dorsal epithelial layer of the wing. Genotypes are as follows: **Ctrl:** MS1096/+; UAS-attp40. ***CHMP1-IR*:** MS1096/+; *UAS-lacZ*/+;UAS-*CHMP1-IR*/+. **hCHMP1B:** MS1096/+; *UAS*-hCHMP1B/+; *UAS-birA*/+. **hCHMP1B-4K>R:** MS1096/+; *UAS*- hCHMP1B-4K>R/+; *UAS-birA*/+**.** **hCHMP1B-ΔMIM:** MS1096/+; *UAS*- hCHMP1B- ΔMIM/+; *UAS-birA*/+. **hCHMP1B *+* *CHMP1-IR*:** MS1096/+; *UAS-dCHMP1-IR/+; UAS*-hCHMP1B**. hCHMP1B-4K>R + *CHMP1-IR*:** MS1096/+; *UAS-dCHMP1-IR/+; UAS*-hCHMP1B-4K>R/+. **hCHMP1B-ΔMIM + *CHMP1-IR*:** MS1096/+; *UAS-dCHMP1-IR/+; UAS*-hCHMP1B- ΔMIM/+.

III. Materials and Methods

1. Molecular Biology

DNA Manipulation and Plasmid Construction

The construct expressing HA-Ubiquitin was obtained from Mathias Treier (Treier et al. 1994). Mammalian expression constructs of human USP8 were cloned by PCR into pmyc-VN155(I152L) vector at KpnI/SalI, and human CHMP1B and mutants were cloned into pHA-VC155 vector at KpnI/SalI. All PCR primers were purchased from Sigma-Aldrich. Full length and truncated GFP-tagged constructs of CHMP1B were kindly provided by Dr. M. Maki (Maemoto et al. 2011). GFP-CHMP1B was used as a template to generate substitution of lysine to arginine (K>R) residues at position K42, K59, K87 or/and K90 by site-directed mutagenesis using the QuikChange II Site-Direct Mutagenesis Kit (Agilent Technologies). GFP-CHMP1B and GFP-CHMP1B-4K>4R mutated for the four lysines cited above were used as a template to generate HA- or VN- and VC- CHMP1B tagged constructs (see suppl. information). FLAG-UBPY construct was used as a template to amplify the fragment at position 3311: 5'-AATCTTCAGCAGCTTATATCC-3' which was cloned into the pSilencer plasmid to generate the silencing construct shUBPY. For the CHMP1B silencing HeLa cells were stably transduced with shRNA non-target, shRNA-CHMP1B TRCN0000159294 (targets 3'UTR region) or shRNA-CHMP1B TRCN0000165547 (targets CDS region) from Mission Sigma shRNA library. No-off target was detected.

2. Cell Biology and Biochemistry

Cell lines and Culture

HEK293T and HeLa cells were purchased from ATTC (LGC Standards, United Kingdom). HEK293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 respectively (Life Technologies) supplemented with 10% heat inactivated fetal bovine serum and 1% Penicillin/Streptomycin mix, and grown in 5% CO2 at 37°C in a humidifier incubator. Serum starvation was performed for 16 hours prior to the stimulation by EGF, TNF or IL1β.

Transfection

HEK293T and HeLa cells transfections were performed at 50% confluence 24 hours after seeding using FuGENE HD (Promega) according to the manufacturer's recommendations at ratio 2:1 respectively. The cells were recovered 24-48h after transfection. Serum starvation was performed for 16 hours prior to the stimulation by EGF, TNF or IL1β.

Bimolecular Fluorescence Complementation (BiFC)

BiFC was used to characterize complex formation between UBPY and CHMP1B proteins in HeLa cells. Cells were seeded at 8 x 10^3 per well in 96-well clear bottom tissue culture plates (#655090, Greiner) and allowed to adhere overnight. Cells were co-transfected with 0.16 μg of Myc-VN-UBPY and 0.04 μg of HA-VC-CHMP1B or mutant. Cells were incubated to allow adequate development of Venus fluorescent protein (VFP) for 24h, fixed in PBS/4% paraformaldehyde for 15 min at room temperature, permeabilized 5 min in PBS with 0.5% Triton-X100. After 1 h saturation in PBS/BSA 1%, cells were immunostained for 1 h with rat anti-HA and mouse anti-Myc primary antibodies diluted at 1/250 and 1/100 respectively in PBS/BSA 1%. To evaluate VFP co-localization with endosomal compartments, rabbit primary antibodies against EEA-1 or Lamp1 were used at 1/400 dilution. Cells were washed 3 times with PBS/ 0.1% Tween-20, then incubated for 1 hour with anti-rat Cy3 and anti-mouse Cy5 secondary antibodies, together with Hoechst 33342 for DNA staining, at a 1/1000 dilution, or with goat anti-rabbit Cy3 and Hoechst 33342 at 1/1000 for co-localization experiment . Cells were washed 3 times with PBS/ 0.1% Tween-20 and PBS/50% glycerol was added before image acquisition, as described in Quantification section.

Subcellular Fractionation

To separate soluble (cytosolic proteins) fraction from insoluble (membrane proteins) fraction, HEK293T cells were harvested, suspended in cold PBSC (supplemented with a protease inhibitor cocktail (Calbiochem) and deubiquitinases inhibitor PR-619 (Sigma-Aldrich) and homogenized by passing cells 20 times through a 26-gauge needle. The supernatant (S) and pellet (P) fractions were obtained by centrifugation at 1000 x g to eliminate cell debris and nucleus and 13000 x g for 20 min at 4°C.The pellets were re-suspended using the same volume of PBS with 1% Triton-x100 and incubating in a shaker at 4°C for 10 min. Insoluble proteins were removed by a 5 min centrifugation at 500 x *g* at 4°C and the supernatant was kept as "the pellet fraction". For the nuclear and cytosolic extraction, NE-PER Nuclear and Cytosolic

Extraction Reagents Kit (#78835; Thermo scientific, France) was used following the protocol provided by the manufacturer.

Sucrose Gradient.

HEK293T cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.5% sodium deoxycholate) and the resulting lysate was layered on top of a series of sucrose steps (40%, 30%, 20% and 10%) and fractionated in a swingingg rotor at 76,000 x g rpm for 17h at 4°C.

Immunoblotting and Immunoprecipitation

Cell lysis was performed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.5% sodium deoxycholate) supplemented with a protease inhibitor cocktail (CALBIOCHEM) and, for the ubiquitination assays, with a pan-deubiquitinases inhibitor PR-619 (Sigma-Aldrich). For co-immunoprecipitation assays, lysates were precleared with Protein A or G-Sepharose beads (Sigma-Aldrich) for 4 hours at 4°C with rotation. Immunoprecipitations were performed in RIPA buffer or PBS.

About 500 μg of total cell lysate were used in the case of GFP IP (anti-GFP; 3 μg); 1 mg of total cell lysate for CHMP1B (anti-CHMP1B; 3μg) and for ubiquitin immunoprecipitation (anti-Ub FK2 6 μg). Immune complexes were precipitated with protein A or G-Sepharose beads with the indicated antibody overnight at 4°C, the beads were washed four times with RIPA buffer and bound proteins were eluted using Laemmli Sample Buffer (BioRad) supplemented with 10% of β-mercaptoethanol and boiled. For ubiquitination assays in presence of transfected HA-ubiquitin, a strong denaturation of lysates was performed before the immunoprecipitation: lysis buffer was supplemented with 1% SDS, the samples were boiled at 100°C for 5 min and then diluted in RIPA to obtain a final concentration of SDS around 0.1%.

Cell lysates were diluted with Laemmli Sample Buffer 4x (BioRad) supplemented with 10% of β-mercaptoetanol, boiled at 95°C for 5 minutes and directly used for immunoblotting.

Western blots were performed as follows: protein lysates and eluates were separated on SDS-PAGE gels of acrylamide of 7.5%, 10%, 12% or 4-15% a (Criterion TGX Stain Free from BioRad). Total amount of protein was detected directly in the gels using ImageLab Stain Free Gel protocol. Proteins were transferred to nitrocellulose membrane or PVDF (BioRad).

Membranes were saturated for 1h in PBS/0.1% Tween-20/5% BSA or skimmed milk, incubated with the appropriate primary antibody for 1 to 3h at room temperature or overnight at 4°C in PBS/0.1% Tween-20/1% BSA or skimmed milk

before 3 washes in TBS/0.1% Tween-20 (PBST) and then incubated an additional hour at room temperature with corresponding secondary antibodies coupled to peroxidase. After 3 rinses with PBST, the membranes were developed with Luminata Forte Western HRP substrate (Millipore) using a Chemidoc apparatus (BioRad). Quantifications were performed using Image Lab (BioRad).

Immunostaining

HeLa cells were washed in ice cold PBS and then fixed with 4% PFA during 15 min at room temperature (RT) before being permeabilized in PBS/0.5% Triton-100 for 5 min at RT. The cells were then washed 3 times in PBS for 10 min at RT and directly processed for saturation using PBS/5% BSA for one hour at RT. Cells were incubated with the primary antibody against UBPY diluted in the PBS/1%BSA at concentration 1/200 over-night at 4°C. For the background noise controls of the secondary antibodies, cells were incubated overnight with the PBS/1%BSA. Cells were then washed 3 times with PBS/0.1% Tween-20 during 10 min at RT with soft agitation then incubated for 1h with the secondary antibody Cy3-labeled anti-mouse at concentration 1/1000 in PBSHS and washed 3 times in PBS/0.1% Tween-20 during 10 min at RT. To label the nuclei, cells were incubated with Hoechst 33342 diluted 1/1000 in PBS for 5 min at RT. Cells were rinsed with MQ water and mounted in DaKo fluorescent mounting media.

Immunostaining of Non-permeabilized cells

HeLa cells were washed in ice cold PBS and then fixed with 4% PFA during 10 min at RT. The cell were then washed 3 times with PBS for 10 min at RT and directly processed for saturation using PBS/5% BSA for one hour at RT. Cells were incubated with the primary antibody against EGFR diluted in the PBS/1% BSA at concentration 1/400 for 1h at RT. For the background noise controls of the secondary antibodies, cells were incubated with the PBS/1% BSA. The cells were washed 3 time with PBS during 10 min at RT with soft agitation then incubated for 1h with the secondary antibody anti-mouse-A488 at concentration 1/1000 in PBS and washed 3 times with PBS during 10 min at RT. To label the DNA cells were incubated with Hoechst 33342 diluted 1/1000 in PBS for 5 min at RT. Then cells were rinsed with MQ water and mounted in DaKo fluorescent mounting media.

Confocal Imaging

The samples were imaged with a 63x magnification (oil immersion) using a Leica TCS-SP2 confocal microscope and the LCS software keeping the same acquisition settings for all the conditions.

Antibodies and Reagents

The following monoclonal (mAb) and polyclonal (pAb) antibodies were used in this study: the mouse mAb against Flag-tag (M2) is from Sigma-Aldrich, the mouse mAb against total ubiquitinated proteins (FK2) is from Enzo, the rabbit pAb against GFP is from Abcam, the rat mAb against HA is from Roche, the mouse pAb against Myc was home-made hybridome supernatant, the rabbit pAb against IStT, CHMP1B (Immunogen: Synthetic peptide corresponding to a region within N terminal amino acids 35-84 of human CHMP1B) and CHMP1A are from Abcam, the rabbit pAb against USP8 (UBPY) and the rabbit mAb against Lamp1 are from Cell Signaling, the mouse mAb against EGFR is from Life Technologies, the rabbit mAb against K48 poly-ubiquitin chains is from Millipore, clone Apu2. The secondary antibodies HRP-coupled goat-anti-mouse, rabbit and rat, and the TrueBlot anti IgG of mice and rabbit are from Invitrogen. The secondary antibodies goat pAb against mouse couple Cy3 or A488 are from Life Technologies. Human EGF recombinant protein was purchased from Sigma-Aldrich. Human TNF recombinant protein is purchased from Sigma-Aldrich and IL-1β is purchased from R&D System.

3. Drosophila Stocks and Manipulation

Fly Stocks

Flies were raised and crossed at 18°C or 25°C using standard procedures. Stocks used for gene silencing are: BL#*28906* (CHMP1-IR) and VDRC#*v107623* (UBPY-IR) and neutralizing UAS transgenes on second and third chromosomes are VDRC#3955 (UAS-LacZ) and VDRC#58760 (UAS-BirA) respectively. The drivers used are MS10096 (BL#8860) and the enGal4 combined with the Notch signaling reporter line (BL#30729).

Transgenic Drosophila Generation (expressing Human CHMP1B constructs)

Constructs encoding human CHMP1B wild type and mutated forms were generated as explained in *DNA Manipulation and Plasmid Construction* and sub-cloned into

pUAST-attb plasmid using EcoR1 and XhoI restriction sites. Stable transgenic lines were generated by injection into the fly stock attP40 in order to integrate each rescuing construct at the same genomic location ensuring similar expression levels- (genotype y1 x67 c23; PattP40). Therefore, putative variability in the amounts of recombinant protein is the results of differences in protein stability rather than to variability of transcription levels. Rescue experiments were then carried out by expressing wild type or mutated constructs of human *CHMP1B* together with *the DmelChmp1*-IR transgene. Rescue experiments were carried out balancing the amount of UAS driving sequences by using VDRC#3955 (UAS-LacZ) and VDRC# 58760 (UAS-BirA) neutral UAS sequences.

Maintenance and Crosses

Fly stock lines are maintained on standard medium at 18ºC or at room temperature. The nutrient medium is composed of 8.3% maize, 8.3% yeast, 1.1% agar, 0.5% moldex (10% methyl-hydroxybenzoate Dissolved in 100% ethanol).

For experimental crosses, 5 to 10 virgin females are crossed with 3 to 8 males. Crosses were maintain at 25ºC (except indications) and flipped every 24h to synchronize the progeny and the phenotypes were compared between progeny of the same age.

Adult Wing Dissection and Mounting for Imaging

Adult wing were dissected in 1xPBS, immersed for 1 minute in 100% ethanol and mounted directly in 50% glycerol 50%1xPBS mounting media. Samples were kept at 4ºC. Images were taken under the stereomicroscope in the following 24h.

Wing imaginal discs dissection and whole-mount staining.

Late L3 larvae wing imaginal disc were dissected in PBS at RT and then fixed with 4% PFA for 15 min at RT before being permeabilized with PBS/0.5% Triton-100 for 5 min at RT, washed 3 times in PBS for 10 min at RT and directly processed for saturation using PBS/5% BSA for 1h at RT. To label the DNA the wing imaginal discs were incubated with Hoechst 33342 diluted 1/1000 in PBS for 5 min at RT then rinsed with MQ water and mounted in DaKo fluorescent mounting media. Genotypes are indicated in the figures legend.

4. Quantification and Statistical Analysis

Densitometry on Western Blots

Quantifications were performed using Image Lab (BioRad). In all the ubiquitination tests, densitometry was performed on the blots and results were expressed as "fold variation over Time 0 or over basal ubiquitination " as they were normalized to the numerical value at time 0 or in non-stimulation or co-expression conditions.

Quantification of plasma membrane EGFR (fluorescence intensity)

Image analysis (cell segmentation and intensity measurements) was done using the open-source software Cell Profiler (Carpenter et al. 2006). The analysis pipeline is available on demand. Statistical analysis was done using GraphPadPrism software as indicated in legends to figures.

Automated acquisition and analysis of cell images

Samples were imaged on an automated ArrayScanVTI microscope (Thermo Scientific) using Zeiss 20x (NA 0.4) LD Plan-Neofluor air objective. Images were collected and analyzed with HCS studio v6.5.0 software. To enable direct comparison, all images within a given experiment were taken under the same magnification and laser intensity settings. Ten fields were systematically acquired for each fluorescent channel (DNA staining, Venus, HA and Myc fluorescence). Venus positive spots and co-localization were quantified using respectively compartmental analysis.V4 and Colocalization.V4 Bio-applications of HCS Studio software, on 150 to 300 cells. For Venus spots quantification, a primary object mask extracted from the Hoechst 33342 channel was created to count nuclei in each image. An enlarged circular mask derived from the primary mask was applied on the HA and Myc channels in order to filter out the negative thus non-transfected cells. A circular mask was then created in the Venus fluorescence channel in order to count Venus spots in transfected cells. Results correspond to the CircSpotCountCh2 feature from one representative experiment out of three. For co-localization, overlap was quantified using the OverlapArea parameter calculated on the bases of the overlap area between Venus spots and indicated endosomal marker. Statistical analysis was done using GraphPadPrism software as indicated in legends to figures.

IV. Discussion and Perspectives

DISCUSSION AND PERSPECTIVES

1. Recall of the main objective and results of the thesis work

Endocytosis of activated receptors requires tight regulation of the components of the endocytic machinery to orchestrate their sequential activation or inactivation that direct the proper sorting of the internalized receptors towards the lysosomes, or their recycling back to the membrane. Among the main mechanisms regulating endocytic flux, we can mention the serial activation of the Rab proteins through the joint action of specific GAPs and GEFs (Stenmark 2009) and the phosphorylation and ubiquitination of Hrs which are necessary for its membrane localization (Gasparrini et al. 2012). Due to their role in membrane remodeling and scission during MVB biogenesis, it is highly relevant to search for new regulatory mechanisms operating directly at the level of the ESCRT-III complexes. Indeed, presently little is known about how human ESCRT-III proteins are regulated and activated *in vivo,* especially by posttranslational modifications, in a context where they undergo a number of crucial conformational changes necessary for their polymerization and assembly at the endosomal membrane.

During my thesis project, we aimed at understanding the function of CHMP1B-UBPY complex in the regulation of receptor sorting and demonstrated the previously uncharacterized direct regulation of the ESCRT-III member CHMP1B by the ubiquitin system.

Starting from the known interaction between CHMP1B and the ubiquitin protease UBPY, we visualized this complex at the level of late endosomes in human cells, determined the extent of the CHMP1B domain mediating the interaction with UBPY and showed that this interaction allows for the co-stabilization of the two partners. Moreover, we evidenced that CHMP1B is ubiquitinated in its helical core and that CHMP1B ubiquitination level is controlled by the ubiquitin protease UBPY. UBPY activity also impacts the capacity of CHMP1B to oligomerize. Ubiquitin linkage to CHMP1B does not alters the total amount of the protein and is not modified by inhibition of the proteasome. It can therefore be hypothesized that CHMP1B ubiquitination is necessary to regulate its function rather than its stability. By *in cellulo* analysis, we have shown that ubiquitin linkage on CHMP1B is induced by EGF, TNFα

and IL1β, whose receptors are known to undergo endocytosis upon activation. CHMP1B ubiquitination in turn regulates EGFR sorting and intracellular trafficking. Using the *Drosophila* model system we have shown that the ubiquitination of CHMP1B is necessary for its function *in vivo*.

We propose that CHMP1B ubiquitination serves as a security lock for spatial and temporal control of CHMP1B function at endosomes probably regulating its oligomerization capacity.

The main results of my thesis are discussed below. Additional results used to discuss the impact of this work can be found in the Annex IV Additional results.

2. Phosphorylation and ubiquitination: two post-translational modifications that may control CHMPs activation and conformational switch

It is widely demonstrated that the ESCRT-III CHMPs undergo conformational switch from open to close conformation that are important for their function. The *in vivo* regulation of the shift from open to close conformation, or reverse, is still largely unknown. By mutational analysis, important domains and amino acid residues implicated in the maintenance of the closed conformation have been resolved for some CHMPs (Lata et al. 2008; Shen et al. 2014; Tang et al. 2015). In other cases, the interaction with a third partner induce the conformational switch (Pineda-Molina et al. 2006; Saksena et al. 2009; Maemoto et al. 2011; Solomons et al. 2011; Shen et al. 2014). But how human ESCRT-III proteins are regulated and activated *in vivo,* especially by posttranslational modifications, and how these posttranslational modifications putatively trigger conformational changes, is poorly known.

As examples, CHMP1A, CHMP1B, CHMP2A, CHMP4B and C and IST1 are phosphorylated by the kinase ULK3 which binds to the ESCRT-III MIM domains. In the case of IST1 and CHMP4B, this phosphorylation is necessary for the regulation of cytokinesis. Specifically, ULK3 phosphorylation of IST1 delays cell abscission by enhancing the interaction of IST1 with the VPS4-LIP5 complex. The function of CHMP1B phosphorylation has not yet been described. However, CHMP1B interaction with ULK3 is necessary for the localization and function of ULK3 in cytokinesis (Maemoto et al. 2013; Caballe et al. 2015; Matias et al. 2015). The recruitment of IST1 and CHMP4C to the points of assembly of ESCRT-III in cytokinesis is induced by their phosphorylation. Caballe and collaborators detected a local increase in the abundance

of both proteins and concluded that phosphorylation of IST1 and CHMP4C may control their *in vivo* polymerization (Caballe et al. 2015).

The crystal structure of IST1-CHMP1B copolymer however does not include any phosphorylation modification (McCullough et al. 2015) and the IST1 mutant defective for phosphorylation is still capable of binding to CHMP1B (Caballe et al. 2015). From these results, and by similarity with the conclusion drawn from my thesis work, the phosphorylation of IST1 might be transient and immediately followed by a dephosphorylation event previously to its entry to the polymer. According to (McCullough et al. 2015), IST1 polymerizes in the close conformation, thus, phosphorylation might trigger the opening of IST1 and dephosphorylation would send it back to the closed conformation needed for polymer formation. In the case of CHMP1A, its phosphorylation inhibits its interaction with the IST1 protein within the ESCRT-III complex (Maemoto et al. 2013). Whether the phosphorylation of CHMP1B similarly inhibits its interaction with IST1 would be of high interest to determine as it could further highlights how the coupling or the uncoupling between phosphorylation and ubiquitination (this thesis) events on a same protein may determine either the hetero-polymerization of CHMP1B with IST1 (McCullough et al. 2015) or in contrast, its polymerization as pure oligomers or hetero-polymers with other proteins than IST1, such as CHMP6 (this thesis).

Another case where phosphorylation may control the conformational change of a CHMP member is CHMP4C which is phosphorylated by the CPC-Aurora B complex in both human cells and *Drosophila* during cell abscission, in an ULK3 dependent manner (Carlton et al. 2012; Matias et al. 2015). CHMP4C phosphorylation by Aurora B targets it to the cytokinesis points (Carlton et al. 2012) but inhibits its activity; the authors proposed that this happened by keeping the protein in a closed conformation (Capalbo et al. 2012). The closed conformation is believed to be the default cytosolic conformation of the CHMPs (except in the case of IST1 which is still a mystery), raising the question why a phosphorylation event would be necessary to maintain this closed conformation upon recruitment to the membranes. One possibility is that pCHMP4C is stabilized in the open state, but that polymerization requires dephosphorylation (similarly as the polymerization of CHMP1B which requires its deubiquitination). The other possibility is that CHMP4C stays in the closed state after its phosphorylation which play a role in the recruitment signal, and also acts as a security lock that will be released by a dephosphorylation event to trigger polymerization. It has been fair well described that opening of CHMPs induces spontaneous membrane binding and polymerization *in vitro*. Thus a security look might be actually necessary to establish an

energetic barrier avoiding spontaneous polymerization in the regions with high concentration of ESCRT-III proteins. This also argues in favor of differential signals, such as phosphorylation and ubiquitination, triggering either ESCRT-III recruitment or their activation/polymerization.
A model in which CHMPs recruit ULK3 and CPC-Aurora B which in turn induces the phosphorylation of IST1 and CHMPs, therefore enhancing the IST1-VPS4 interaction and inhibiting the IST1-CHMPs interaction and delaying the abscission has been proposed as a check-point for cytokinesis. Consecutive dephosphorylation of IST1 and CHMPs would trigger IST1-CHMP1B polymerization, membrane scission and termination of cytokinesis (for a putative model of membrane scission see Fig. D3). However, the situation is far from being so simple, since for the moment, at least the two different kinases cited above are implicated in this process and both of them undergo very complex regulation of their localization and activity. What are the sensory mechanisms deciding to trigger or not membrane scission at the end of cytokinesis process are still not fully understood.

In this thesis work, we have shown for the first time the ubiquitination of an ESCRT-III protein. In overexpression experiments we have identified that CHMP1B is mainly monoubiquitinated. The observation of higher molecular weight bands may reveal the existence of multi-mono-ubiquitination events or alternatively, of ubiquitin chains. In the presence of proteasomal blockers the ubiquitination status of CHMP1B did not significantly change and analyses of the immunoprecipitated GFP-CHMP1B with anti-K48 antibodies showed no specific signal. Therefore, the high molecular weight bands would correspond to yet unknown polyUb linkage on CHMP1B other than K48 chains. Presently, we do not know either whether the major mono-ubiquitinated form of CHMP1B results from the linkage of one ubiquitin moiety or alternatively from the processing of polyUb chains by a specific DUB.

Using single point mutation of lysine residues of CHMP1B, we have identified K87 and/or K90 situated in the linker region connecting α-helices 2 and 3 of CHMP1B as the major site(s) of ubiquitination. Interestingly these two lysines are closed to each other. Compared to the human protein, in *Drosophila* the K87 is conserved but not the K90, while in *Arabidopsis* the K90 is conserved but not the K87 (Annex Fig. A5). This argues in favor of the importance of keeping at least one lysine residue in that region, that can undergo ubiquitin linkage independently from each other, thus triggering the ubiquitination of the protein. This linker region is crucial for the conformational changes that occur between the closed and open conformations in ESCRT-III proteins

(Fig. D1). Notably, in the conversion from the close to the open conformation, the flexible linker region adopts a helical structure allowing association of α-helices 2 and 3 in a continuous helix (McCullough et al. 2015; Tang et al. 2015).

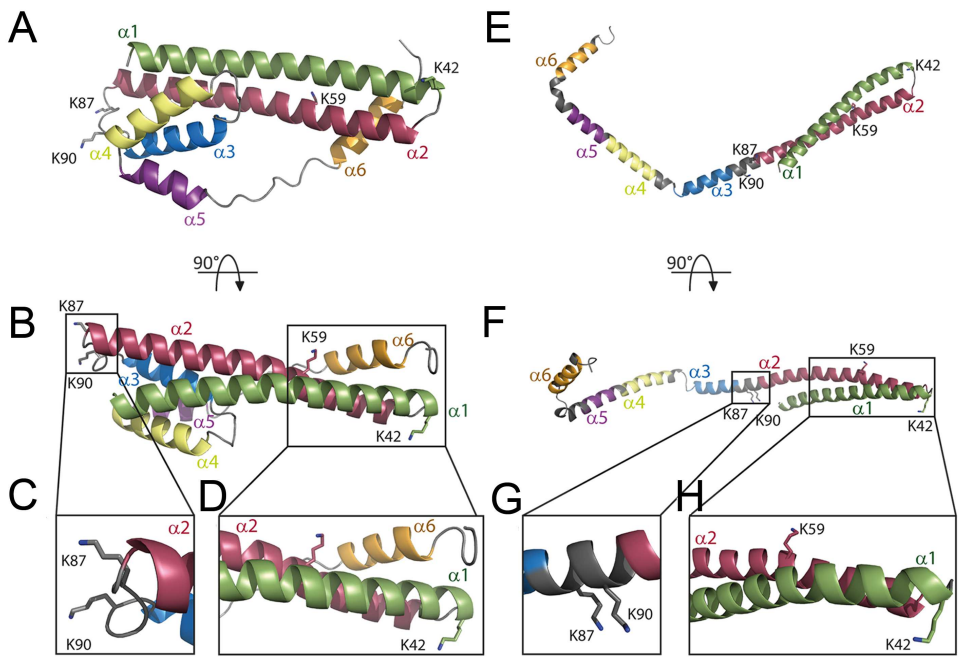


Figure D1: CHMP1B conformation switch. **A** Structural model of CHMP1B in its closed conformation. **B** Side view of the CHMP1B closed conformation. **C, D** Close-up views showing the positions of lysines (K42, 59, 87, 90) possibly implicated in ubiquitination. **E** structure of the CHMP1B open conformation present in the CHMP1B polymer. **F** Side view of the CHMP1B open conformation. **G, H** Close-up views of the putatively ubiquitinated lysines in the open conformation. **A-H** Lysine residues are shown as sticks. Alpha helices are numbered and colored as indicated (Carmen Aguilar-Gurrieri).

Several observations led us to suggest that ubiquitination of either K87 or K90 (or both) residues favors the transition from the "closed" to the "open" state or stabilizes the open conformation of CHMP1B.

We first demonstrated the existence of endogenous monomeric, dimeric or oligomeric forms of endogenous CHMP1B and observed that endogenous

ubiquitinated CHMP1B exist as monomer or dimers but not as oligomers. Then, we showed that non-ubiquitinated monomers and dimers do not expose the auto-inhibitory interface to a specific antibody for this epitope, while this region is accessible in the ubiquitinated CHMP1B monomers and dimers. We concluded from these observations, that in the ubiquitinated status, CHMP1B may be found in an open conformation, or at least in a conformation where this loop is exposed for recognition by the antibody. The recognized epitope is also free for antibody binding in high molecular complexes (oligomers) which is in accordance with the fact that CHMP1B is found in the open conformation in polymers (McCullough et al. 2015).

This "oligomeric form" of CHMP1B was never found in the pool of the ubiquitinated proteins within cell extracts. Thus, taken together, our results show that ubiquitinated CHMP1B is in a conformation that exposes the auto-inhibitory surface therefore arguing in favor of an "open or active" conformation which cannot polymerize, while the non-ubiquitinated monomers and dimers would be in a close conformation (Fig. D2).

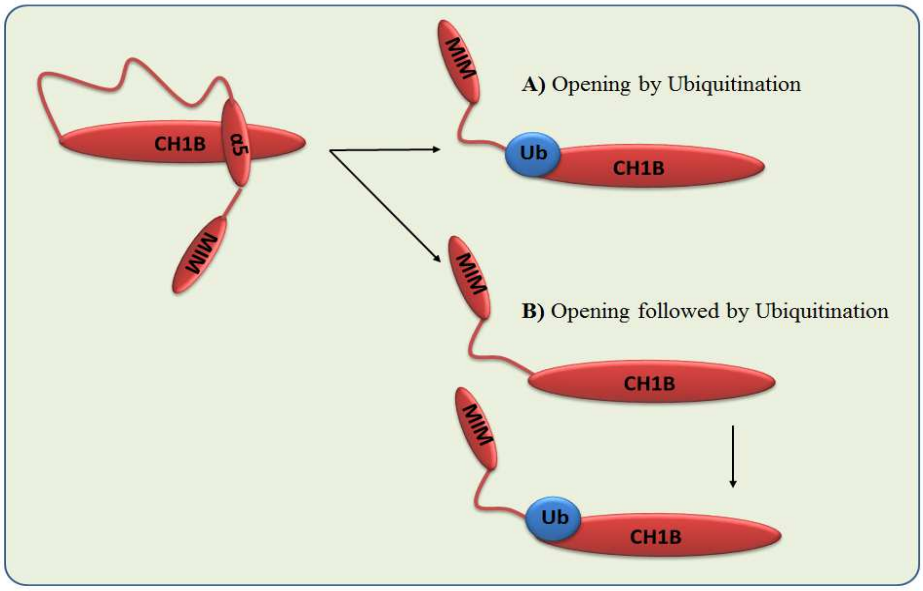


Figure D2: Models for CHMP1B conformational change controlled by ubiquitin. A: Opening of CHMP1B is induced by ubiquitination. **B:** once opened by other meaning, CHMP1B is ubiquitinated to stabilize this conformation.

We thus hypothesized that a deubiquitination step is needed for the entry into the complexes and that ubiquitination of the subunits may serve as a security lock during some kind of check-point during the process of ILVs formation.

Although at this point we are not in a position to define further the structural changes taking place, the ubiquitination of CHMP1B is undoubtedly implicated in conformational changes that govern the oligomerization capacity of CHMP1B. The extremely different structural models of interaction and assembly that have been described for otherwise very similar CHMPs make very difficult to propose an assembly model for CHMP1B, and in any case, is an objective beyond this study.

Further mechanistic and structural studies will be also necessary to fully understand how the attachment of an ubiquitin moiety induces and/or blocks CHMP1B in an open conformation and why the ubiquitinated CHMP1B is unable to polymerize. The production of in vitro Ub-CHMP1B would be a very interesting strategy to study and confirm putative conformational changes induce by ubiquitin linkage of CHMP1B. This would imply however, the determination of an E1, E2 and E3 ligases set that could trigger CHMP1B ubiquitination *in vitro* and the realization of ubiquitination assays on purified CHMP1B.

In addition, further proteomic analysis of immunoprecipitated Ub-CHMP1B would be of high interest to identify the ubiquitinated lysine residues by mass-spectrometry but stays challenging due to the relative low abundance of Ub-CHMP1B compared to the pool of non-ubiquitinated CHMP1B.

3. Investigation of the nature of CHMP1B "oligomers"

As mentioned above, endogenous CHMP1B is detected by Western blot analysis of cell extracts at three bands of different molecular weight that would correspond to monomers, dimers and oligomers. At this stage of our study, we cannot conclude however whether those CHMP1B oligomeric forms contains other tightly associated partners, notably from the ESCRT machinery. To answer this question, complementary experiments were conducted. Western blotting of cell lysates with CHMP1B, CHMP1A, CHMP2A, CHMP3, and CHMP6 failed to reveal a band at the same molecular weight of ~200 kDa seen with the CHMP1B antibody (Annex Fig. A6). However this experiment is not fully conclusive as hetero-polymers may not be detectable or not SDS-

resistant. Interestingly, separation of CHMP1B dimers and oligomers on a sucrose gradient after cell lysis revealed the presence of IST1 in at least one fraction containing CHMP1B oligomers suggesting a possible association of IST1 to these oligomers of CHMP1B.

In any case, if hetero-interaction happen with IST1 and/or CHMP6 and/or other partners, as surely is the case from previous reports, the strength of those interactions is not sufficient to resist SDS- denaturation as in the case of the CHMP1B intracellular species. Specifically, determining why those species exist in the cells in the case of CHMP1B could enrich the actual knowledge on the mechanism of assembly of and membrane scission by the ESCRT-III machinery and a possible model is shown below in the Figure D3.

Other open questions remain to better understand the dynamics of assembly of ESCRT-III proteins such as: how CHMP1B or Ub-CHMP1B are recruited to the ESCRT-III complexes at the endosomal membrane? How the machinery manages to include the IST1-CHMP1B polymer in the architecture of the complex when they have membrane binding affinities positioned in opposite topology from the rest of the ESCRT-III filaments? Is ubiquitination of CHMP1B also a localization signal like phosphorylation in the case of other CHMPs? Which E3 ligase triggers CHMP1B ubiquitination and what is the molecular cascade triggering its activation in response to EGF? Is there a signal for deubiquitination? These questions need to be addressed using wild type or CRISPR/Cas9 modified endogenous proteins or using non-tagged constructs that we could be properly visualized *in vivo* and differentiate from the wild type protein.

4. Regulation of CHMP1B by UBPY and its potential implication for ESCRT-III assembly

4.1 CHMP1B and UBPY only interact in the cell context

The interaction between UBPY and CHMP1B was described in human cells previously to the start of my thesis (Row et al. 2007). In their study, the author showed that the MIT domain of UBPY was necessary for the interaction *in cellulo* with CHMP1B as well as for the UBPY endosomal recruitment by association with ESCRT-III members. But no further study on the minimal domains of CHMP1B needed for the

interaction were described. Based on the structural similarity between CHMP1B and CHMP3 and the nature of the interaction between CHMP3 and AMSH (Solomons et al. 2011), we initially hypothesized that the interaction with UBPY would happened through the MIM domain of CHMP1B.

One aspect of the initial project was actually to study the interaction interfaces between UBPY and CHMP1B in collaboration with Pr. Weissenhorn's group who first described the structural interface between CHMP3 and AMSH. To this end, efforts were made to purify the complex in order to obtain structural data by crystallography using the N-terminus of UBPY (corresponding to the dimerization region and comprising the domain MIT) and the MIM domain of CHMP1B. Dr. E. Poudevigne notably tried to co-purify the N-terminal regions of UBPY (amino acid residues 8-138) and C-terminal of CHMP1B (amino acid residues 126-199). However, the tests carried out on a gel filtration column revealed the absence of a UBPY-CHMP1B complex and further biochemical assays conducted failed to reveal strong interaction between the two constructs.

Since UBPY includes within its N-terminus a dimerization domain that may prevent the interaction of UBPY with another partner, Dr. E. Poudevigne and Dr. AC. Jacomin generated a construction of UBPY which can no longer form dimers by removing the region corresponding to the first helix (amino residues 18-29), or by mutating the amino acids of UBPY at the dimerization interface, in order to obtain a monomeric protein in solution. This strategy however still failed to allow purification of the complex. Finally, Dr. C. Aguilar-Gurrieri tried with GST-pull down, surface plasmon resonance (SPR), isothermal titration calorimetry (ITC) and size exclusion chromatography-multi-angle light scattering (SEC-MALS) techniques using different constructions of both proteins. However the direct interaction *in vitro* has been elusive until the moment.

During my thesis, I focused on the *in cellulo* analysis of the complex. Using co-immunoprecipitation experiments, I mapped the interaction region between CHMP1B and UBPY to residues 105 to 199 of CHMP1B which contains the helix α4, 5 and 6. This region thus extends beyond the MIM domain and largely overlaps with the one described for the interaction between CHMP1B and Spastin that involves the residues 148-199 of CHMP1B (Yang et al. 2008). Furthermore, Dr. C. Aguilar-Gurrieri confirmed this result in HeLA cells using CHMP1B constructs lacking the helix α6, the helixes α5-6, or the helixes α4, 5 and 6. The deletion of only the helix α6 of CHMP1B -

that contains the MIM domain- or of the two helixes α5-6 did not significantly impaired the interaction between the two proteins, while the deletion of the helixes α4, 5 and 6 did.

Moreover, we have observed stabilization of endogenous UBPY upon overexpression of CHMP1B in HeLa cell, co-stabilization of both overexpressed proteins in HEK293T cells by western blot analysis and increase or stabilization of the oligomeric fraction of endogenous CHMP1B upon overexpression of UBPY in HEK293T cells (Fig: S1A,R1C and R3D). These observations are consistent with the two proteins interacting *in vivo*, as overexpression of one partner could very well alter the half-life of the other partner. They are also in agreement with UBPY affecting the oligomerization of CHMP1B. Whether CHMP1B mutant lacking helixes α4, 5 and 6 is able to be recruited to the membranes and how this influences the recruitment of UBPY to the same compartment will need to be addressed using non-tagged constructs or modifications of endogenous CHMP1B.

Therefore based on all the results *in cellulo* and the impossibility to detect a direct interaction *in vitro,* we conclude that putatively a post-translational modifications not present in bacterial expressed proteins, or a third protein are needed for the interaction of both proteins. In the future, *in vitro* induced ubiquitination of purified CHMP1B would allow to test if the ubiquitin moiety on CHMP1B triggers its association with UBPY. In addition, proteomic analysis of the complex purified from human cells may also help in the identification of other indispensable partners.

4.2 Role of UBPY-dependent regulation of CHMP1B in ESCRT-III assembly

From previous studies, it was known that through its interaction with the CHMPs, UBPY is recruited to the endosomes, permitting its interaction with STAM (Niendorf et al. 2007; Row et al. 2007). The group of Dr. Sylvie Urbé showed that in cultured human cells, the depletion of UBPY reduced the total amounts of Hrs and STAM by 50% and 90% respectively, while the ubiquitinated forms of STAM accumulated (Row et al. 2006). Similar observations have been made by Sandra Niendorf and colleagues in mice (Niendorf et al. 2007). Moreover, STAM is able to stimulate UBPY DUB activity *in vitro* (McCullough et al. 2006). However Row and colleagues showed that CHMPs did not stimulate the DUB activity of UBPY *in vitro (Row et al. 2007)*. Similarly, CHMP3 does not stimulate AMSH DUB activity in the same

kind of assay (McCullough et al. 2006). Therefore, the function of the interaction of either AMSH or UBPY with the ESCRT-III proteins described so far consists in the endosomal recruitment of these enzymes. This recruitment is proposed to facilitate the deubiquitination of other partners such as HRS, STAM and endocytosed receptors.

In this study however, we have shown that CHMP1B is itself ubiquitinated and that it is not only a partner but also a substrate for UBPY. We have shown that overexpressed UBPY is capable of deubiquitinating CHMP1B *in vivo* while *Ubpy* silencing induced the accumulation of ubiquitinated CHMP1B. The expression of the catalytic dead mutant also led to the accumulation of ubiquitinated CHMP1B. Hence, constitutive ubiquitination of CHMP1B exists in the steady-state and is regulated by UBPY.

For the moment, we cannot rule-out that other DUBs than UBPY may control the level of CHMP1B ubiquitination. It would be interesting to test if AMSH regulates CHMP1B ubiquitination as it contains a MIT domain and is also recruited to the ESCRT platform.

In their study, Row and colleagues showed that CHMP1B levels were not altered upon silencing or overexpression of *Ubpy in vivo*. However, in the lysates they have only analyzed the monomeric fraction of endogenous CHMP1B by Western blot. Overexpression of UBPY in our hands induces or stabilizes the endogenous CHMP1B oligomeric species suggesting that UBPY dependent removal of ubiquitin moieties favors CHMP1B oligomerization (Fig. R3D and graphical abstract in the general conclusion).

In line with these results indicating that CHMP1 is regulated by UBPY, or at least by a deubiquitinating enzyme, we also showed that ubiquitination of CHMP1B is dynamically regulated in response to EGF or cytokines stimulation at specific kinetics depending on the endocytic signal (see also below). As visualized in the membrane fraction, endogenous ubiquitinated forms of CHMP1B rapidly accumulate upon EGF stimulation (at 5 min) and then the ubiquitinated forms of CHMP1B rapidly decrease to reach the basal level (at 15 min). Since the total amount of CHMP1B analyzed did not vary, this fluctuations are likely due to active ubiquitination and deubiquitination cycles of CHMP1B most probably in association with its recruitment to the membranes, were CHMP1B probably is part of a complex containing a yet unknown E3 ligase and UBPY.

Consistent with a role of UBPY on CHMP1B activation at the membrane, using a Venus complementation assay in human cells and High Content Screening (HCS) analysis, we have shown that the two CHMP1B and UBPY partners interact on Lamp1 positive late endosomal compartments. This result is also in agreement with the implication of CHMP1B in MVB biogenesis and receptor sorting at the later steps of the ILVs (Spitzer et al. 2009; Stuffers et al. 2009). Using this system however, we did not find any co-localization with Rab7 positive endosomes where Rab7 is a marker for transition endosomes from early to late endosomes (Chavrier et al. 1990). The immunostaining of the cells with the Rab7 antibody was not optimal (not shown). Therefore, we analyzed the localization of CHMP1B using the *Drosophila* FLPout system in the fat body (See Annex I.C for the description of the system) to generate clones of cell expressing the fusion protein GFP-Rab7. We stained larval fat body with anti-CHMP1B antibody and confirm the absence of co-localization of CHMP1B with the Rab7 positive endosomes (Fig. A7). Therefore, the CHMP1B-UBPY complex is likely not present in this compartment.

Consequently, we place the CHMP1B-UBPY interaction at the very late steps of the formation of the ILVs in association with the Lamp1 positive endosomal membranes. UBPY is also recruited at late steps of cytokinesis at the level of the central spindle via IST1 and CHMP1B (Mukai et al. 2008). During cytokinesis, silencing of *Ubpy* leads to the accumulation of ubiquitinated proteins in the cleavage regions and generate defective abscission. It is therefore possible that the recruitment of UBPY to the central spindle is also MIT and CHMP1B interaction-dependent like in the case of Spastin (Yang et al. 2008) and that members of the abscission machinery are regulated by ubiquitination-deubiquitination cycle (Mukai et al. 2008) just as in the case of regulation of any protein by phosphorylation-dephosphorylation.

Interestingly, in a very recent study, Johnson and colleagues show that Doa4 (the yeast UBPY) interaction with Vps20 (the yeast CHMP6) at the beginning of the ESCRT-III filament assembly inhibits cargoes deubiquitination (Johnson et al. 2017), Once the cargoes are trapped into the ESCRT-III polymer, Doa4 is released from Vps20 and binds Bro1 (the yeast Alix). The association of Doa4 with Bro1 then promotes Doa4 catalytic activity and stabilizes ESCRT-III polymer by inhibiting its disassembly. In this way escape of cargoes is prevented by inhibition of their premature deubiquitination.

This hypothesis was mostly drawn from the fact that ESCRT-III complexes accumulate upon expression of either the wild-type or the catalytic dead mutant of

Doa4. However, these complexes displayed much higher molecular weight in the case of the wild-type form of the enzyme. Therefore, Doa4 catalytic activity would be necessary at the later steps of ESCRT-III filament assembly while earlier steps of ESCRT-III filament assembly may be favored by Doa4 expression, independently of its catalytic activity. The authors also performed rescue experiments using ESCRT-III or ESCRT-II mutants. However, they did not tested the effect of mutation of late ESCRT-III members acting after the Doa4 interaction shift from Vps20 to Bro1, such as for example for IST1 or CHMP1B. In addition Vps4 has been very recently implicated in the control of dynamic ESCRT-III subunits turnover which is necessary to sustain efficient net growth of ESCRT-III filaments during the scission process (Mierzwa et al. 2017).

From this two recent studies together with my thesis results, I can propose a slightly different hypothesis for UBPY function in human cell system than the one proposed before (also presented in the graphical abstract in the general conclusion) in which the deubiquitination of CHMP1B at the late stage of ESCRT-III assembly would promote CHMP1B assembly into the membrane-associated ESCRT-III polymer. In this case, in UBPY overexpressing cells the accumulation of oligomers that we observe could be the either the result of the inhibition of CHMP1B depolymerization by deubiquitinating recent depolymerized monomers which would be send back to the filament or as proposed before, the result of the enhancement of the continuous entry of new cytosolic monomers.

In the hypothesis where UBPY favors CHMP1B entry in the ESCRT-III polymers, in a wild type situation, deubiquitination of CHMP1B would allow its entry into the polymer and may occur only at late steps, when UBPY catalytic activity is activated by Bro1.

In the particular case of the IST1-CHMP1B co-polymer, it presents different membrane binding architecture than the rest of the CHMPs polymers and the shift in the direction of the membrane attracting forces could help in the final step of bringing the membranes to very close contacts which will generate the scission. To do this, the attraction forces between activated CHMP1B subunits has to be high enough to attract one another and drag the membrane into the pore formed by IST1-CHMP1B polymer (see model for hemi-fusion and scission generated by IST1-CHMP1B below). This requirement could explain the existence of SDS-resistant species of CHMP1B

oligomers. Based on the mild phenotypes produced by the silencing of *chmp1b* alone, probably other CHMPs act in a similar manner with IST1.

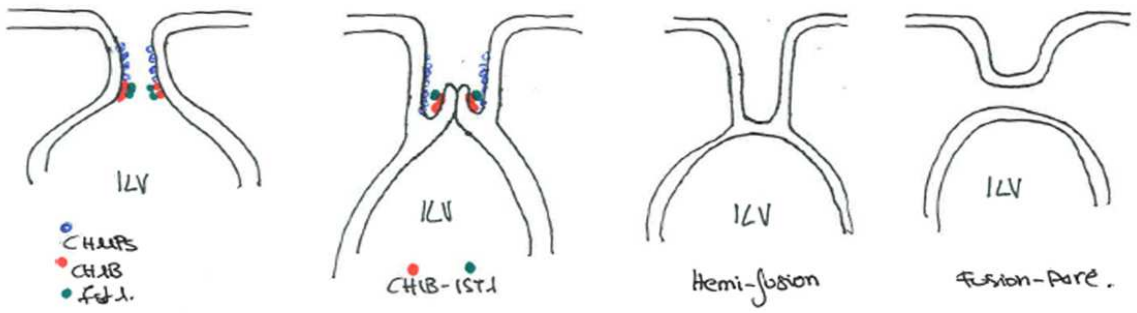


Figure D3: **Model for scission** generated by assembly of the CHMP1B-IST1 polymer in the necks of the forming ILVS. Based on (Fuhrmans and Muller 2015) model for dynamin mediated bi-lipidic membrane fusion.

It would be also interesting to evaluate the kinetics of the interaction of UBPY with CHMP1B following stimulation with EGF. This would allow us to correlate the deubiquitination of CHMP1B between 10 and 15 minutes of EGF stimulation with a putative recruitment of UBPY within a protein complex containing CHMP1B further validating the functionality of this interaction. However, since it has been shown that UBPY can be recruited by other ESCRT-III MIT domains, it would be difficult to differentiate which proteins of the ESCRT mega-complex are directly interacting with UBPY. The study of the kinetics using the Venus system is not useful, since Venus protein reconstitution is not reversible. Pulse-chase of EGF in combination with chemical cross-linking and biochemical analysis, HRTF or Super-resolution microscopy techniques could be useful to further investigate this question in living cells.

In this study we have demonstrated that CHMP1B is monoubiquitinated. We have shown that UBPY is able to deubiquitinate CHMP1B. This is consistent with the fact that UBPY is capable of hydrolyzing K63-, K48- polyUb and monoubiquitin *in vitro* (Mizuno et al. 2005). Whatever the nature of the ubiquitin moiety linked to CHMP1B, We have further investigated the requirement of this modification for CHMP1B function in cell trafficking and *Drosophila* wing development (see next paragraphs) and

let the question of the CHMP1B assembly mechanisms open that may be solved in the future by proteomics and structural analyses.

5. Functional evidences for the role of CHMP1B and CHMP1B ubiquitination in EGFR trafficking

As mentioned in details in the introduction, cell stimulation by EGF induces EGFR internalization and its active sorting at the endosomes where it can be either directed to the lysosomal degradation pathway or recycled back to the plasma membrane (Tomas et al. 2014). The high doses of EGF used in this study trigger the ubiquitination of the EGFR at the PM inducing the CIE of the EGFR and its subsequent degradation though the MVB pathway (Haglund et al. 2003; Mosesson et al. 2003; Huang and Sorkin 2005; Sigismund et al. 2005; Sigismund et al. 2008) (Fig. 26). A number of studies demonstrated the requirement of members of the ESCRT-III family in the sorting process (Row et al. 2007; Baldys and Raymond 2009; Jones and Rappoport 2014). Several functions have been actually described for CHMP1B in particular. The yeast Did2 protein (CHMP1) acts in concert with Ist1 in the control of Vps4, which disassembles ESCRT-III polymers at a late stage of MVB sorting (Nickerson et al. 2006; Dimaano et al. 2008; Rue et al. 2008). Furthermore, CHMP1B would be implicated in the formation of recycling tubules from endosomes by stabilizing positively curved membrane tubules (McCullough et al. 2015). However, as discussed in the introduction, most of the hypotheses in human system are issued from results obtained with protein overexpression while few phenotypic defects induced by loss of function of CHMPs encoding genes have been reported, putatively due to functional redundancy and/or incomplete gene silencing.

Here, we show for the first time the functional requirement of CHMP1B for EGFR trafficking. We used stable shRNA mediated silencing of c*hmp1b* in HeLa cells and actively searched for specific defects in EGFR expression. The immunostaining of non-permeabilized cells notably allowed to detect that the EGFR persisted at the surface of *chmp1b*-silenced cells at 60 min of EGF stimulation, i.e., at a time where the EGFR was undetectable at the surface of control cells. Nevertheless, the EGFR was properly internalized in *chmp1b*-silenced cells. These observations are consistent with a defect in EGFR sorting putatively causing an enhancement of EGFR recycling rate back to the plasma membrane. This phenotype could be the cause or the consequence of a

decreased in the lysosomal degradation. Complementary biochemical analysis were conducted and a delayed degradation of EGFR upon stimulation by EGF was indeed detected in *chmp1b*-silenced cells, suggesting a reduction of EGFR lysosomal degradation rate (Fig. A8). In these experiments, the *chmp1b*-silenced cells presented higher levels of EGFR expression in the steady-state (i.e., in non-stimulated cells) indicating that CHMP1B is somehow also implicated in the maintenance and turnover of the basal levels of cellular EGFR. This feature makes even more difficult to fully interpret the impact (and determine the molecular causes) of the variations kinetics in EGFR membrane disappearance and degradation after EGF stimulation in *chmp1b*-silenced cells compared to control cells. Particularly, as described in the introduction (chapter 2 and 4), quantitative differences in the concentration of EGFR at the PM can trigger different cellular responses to a same signal. Nevertheless, our observations clearly demonstrate the requirement of CHMP1B for proper EGFR turnover and trafficking in human cells.

Having identified a phenotypic defects caused by the knockdown of *chm1b*, we further showed that the wildtype form but not an ubiquitin-defective mutant of CHMP1B rescued the kinetics of both EGFR disappearance at the membrane and of EGFR degradation (Fig. R4A and additional Fig. A8), thus demonstrating the functional relevance of CHMP1B ubiquitination. Moreover, the fact that CHMP1B ubiquitination is dynamically regulated by EGFR also argues in favor that ubiquitin linkage plays a physiological important function. Actually, we showed that upon EGF stimulation endogenous non-ubiquitinated CHMP1B is found mostly in the cytosolic fraction while ubiquitinated CHMP1B strongly and transiently accumulates on membranes at 10-15 minutes of EGF stimulation. Therefore we correlated the transient recruitment of ubiquitinated CHMP1B in the membranes with the induction of the EGFR dependent signaling pathways and its trafficking towards the lysosomes. This correlation implies that an E3 ligases targeting CHMP1B is activated following EGF stimulation. Putative candidates are the E3 ligases recruited on the phosphorylated EGFR such as Cbl. Alternatively, E3 ligases and DUBs are known to act in concert in multimeric complexes, therefore, an E3 ligase interacting with UBPY, such as Nrdp1, may trigger CHMP1B ubiquitination (see introduction chapter 4).

Consequently, EGFR signaling directly or indirectly generates an auto-regulatory loop through the ubiquitin-dependent regulation of CHMP1B, as also shown for other proteins of the endosomal machinery. Although the molecular mechanism that governs

CHMP1B ubiquitination is still unknown, this work reports for the first time the EGFR-dependent dynamic ubiquitination and deubiquitination cycle of a CHMP protein.

In addition, the differences in the ubiquitination dynamics of CHMP1B depending on the nature of the signaling molecules (EGF, IL1β or TNFα) could indicate the possibility that CHMP1B ubiquitination is part of an ubiquitin-dependent signaling sensing mechanism that would differentially control the dynamics and the decision fate between recycling or degradation among different membrane receptors. Similarly as for the CIE versus CME of cell surface receptors, this ubiquitin-dependent signal would transform the receptor phosphorylation events in a "on-off" responses through the induction or not of membrane scission by the ESCRT-III machinery (Introduction chapter 4 Fig. 26).

At this stage of our studies, the most appropriate strategy to further test and describe in details how posttranslational modification of CHMP1B influences its activity would be first to reach a better understanding of its functions in membrane remodeling and scission, notably by using CRISP/Cas9 induced endogenous mutants. In order to discriminate CHMP1B specific function in cytokinesis or endocytosis, it might be also interesting in the future to synchronize the cell cycle of the cell population under study.

Finally, it would be highly relevant to investigate whether other CHMPs are regulated by the ubiquitin system and if this is the case, whether a similar dynamic upon various cell stimulation signals can be observed.

6. Functional Relevance of CHMP1 ubiquitination *in vivo*

The crucial role of CHMP1 ubiquitination was further confirmed *in vivo* by analyzing the wing development of mutant and transgenic *Drosophila melanogaster* flies lines. Actually, proper wing development depends on signaling molecules, such as Hedgehog (Hh), Wingless (Wg) or Vein (Vn), the ligand for EGFR or Notch, which activate signal transduction pathways through endocytosis of their receptors (Ma et al. 2007; Matusek et al. 2014; Pradhan-Sundd and Verheyen 2014; Valentine et al. 2014; Conner 2016). This makes wing development an ideal system to evaluate the contribution of a protein to endocytosis, even if, in the context of an entire organ, the

observed phenotypes may also results from defects in other cell processes, such as in the particular case of CHMP1: cytokinesis and exo-vesicles formation.

In *Drosophila*, ubiquitous silencing of *Chmp1* or *Ubpy*, -that are respectively orthologous to human *chmp1b* and *Ubpy/Usp8-,* is lethal before the pupal stage (our unpublished observations) indicating that both proteins are essential for *Drosophila* development. Therefore, we have generated and used transgenic fly lines expressing silencing hairpins under the control of UAS promoters to induce silencing of *Chmp1* or *Ubpy* only in the wing tissue (see UAS-Gal4 system in Annex I.B). Using the driver line MS1096-Gal4, we induced the silencing of these two genes, alone or in combination, in the dorsal epithelium of the wing tissue. In these conditions individual *Chmp1* or *Upby* silencing produced a similar severe developmental phenotypes with the wing slightly curved forward, due to the asymmetry in the expression of the Gal4 protein along the DV axis. In the double mutant, we observed a synergistic effect resulting in no wing formation. As protein depletion is usually incomplete in gene silencing experiments, the observed synergistic defect suggest that both proteins act in common in processes leading to wing formation, reinforcing the idea that UBPY is required for the regulation of CHMP1B function.

Using the same Gal4 driver line, we ectopically expressed wild-type or mutated forms of the human CHMP1B in the *Drosophila* wing together or not with the *Chmp1* silencing transgene. Since it was previously reported than the tagged CHMP1 protein was not fully functional in *Drosophila* (Valentine et al. 2014), we used untagged proteins to ensure that the protein would be functional. Wing directed expression of the wild type human CHMP1B fully rescued the wing defects induced by *Chmp1* silencing, while expression of the ubiquitin-deficient form of human CHMP1B *(4K>R)* only very partially suppressed these wing defects. Partial rescue by the ubiquitination-defective mutant indicates that modification of CHMP1B by ubiquitin linkage contributes to the protein function in *Drosophila* and provides evidence for the evolutionary conservation of an ubiquitination-based regulation of CHMP1 proteins.

In these experiments, we observed that ectopic expression of the wild-type or ubiquitin-defective forms of the human protein in the wing produced no phenotype, indicating that the corresponding products were not deleterious to wing developmental processes. Thus, CHMP1B is not trapping a limiting factor in any of the processes in which the endogenous *Drosophila* CHMP1 protein participates. Furthermore, the mutation of the four Lysine to Arginine residues did not produced a dominant negative protein, suggesting a conserved structural architecture of the ubiquitin-defective mutant.

In contrast, the expression of the truncated human CHMP1BΔMIM caused similar phenotype as *Chmp1* gene silencing, meaning that CHMP1BΔMIM dominantly prevents endogenous CHMP1 function. We suggest that CHMP1BΔMIM traps important partners of CHMP1 thus acting as a dominant-negative form of the protein. Alternatively, it may polymerize and form toxic aggregates within the cells. In any case, our results confirm *in vivo* some previous studies showing that the MIM domain of CHMP1B is essential for its function. Indeed, as expected from its dominant effect, this construct was not able to rescue the wing defects induced by *Chmp1* silencing (Fig. S6).

We have then performed similar experiments using the eye-specific GMR-Gal4 driver line (Ray and Lakhotia 2015). However, in this tissue, the silencing of *Chmp1* did not produce any phenotype (not shown) suggesting that CHMP1 is not strictly required for eye development. The differences in the requirement of CHMP1 for the development of different organs may reflect differences in the nature of the developmental signals triggering tissue differentiation that highly depends on secreted morphogens in the case of the wing but not, or much less, in the case of the eye development. Interestingly however, the eye-directed expression of the human CHMP1BΔMIM (but not of CHMP1B and CHMP1B-4K>R) produced strong morphological defects indicating that it can acts as dominant negative protein also in this organ, most probably by affecting a number of processes in addition to endocytosis (Fig. A9).

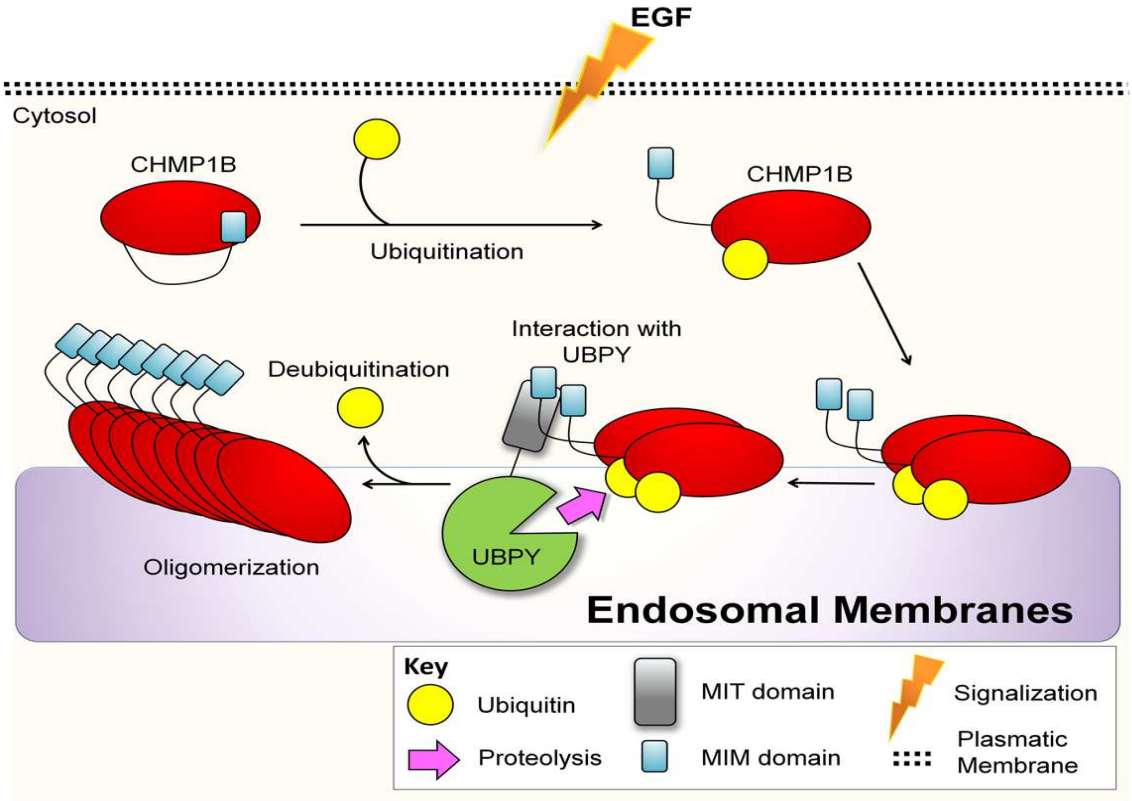
Remarkably, a complex set of signaling events establishes a single row of active Notch pathway cells in the wing larval imaginal disc, at the level of the future wing margin (Irvine and Vogt 1997; Irvine 1999) and see Annex II. Using a EGFP reporter gene expressed under Notch Responsive Elements (NRE), one can therefore detect Notch activation in the future wing margin by simple EGFP detection by confocal analysis of dissected wing imaginal discs, in various genetic backgrounds. Using the wing driver *engrailed (en)*-Gal4 to induce the silencing construct in the posterior part of the imaginal wing disc in flies carrying this NRE-EGFP reporter construct (see Annex I.D), we observed that silencing endogenous *Chmp1* resulted in an enlarged domain of EGFP expression both in the domain of En expression and slightly beyond (Fig. R5C). This domain was restored to a single row of EGFP expressing cells by expressing the human wildtype CHMP1B but not its ubiquitin-defective version. Thus, defective ubiquitination of CHMP1B impairs its function in the establishment of a restricted domain of Notch activation at the wing margin.

The fact that enlarged domain of Notch activation is detected beyond the domain of en-Gal4 expression (i.e; in the anterior part of the wing) reflects a cell non-autonomous effect which is consistent with previous studies showing the role of CHMP1 in long range signaling events (Valentine et al. 2014) that in turn governs the establishment of the Notch signaling domain (Cadigan 2002; Zecca and Struhl 2002a; Zecca and Struhl 2002b; Rives et al. 2006; Vaccari et al. 2009; Aoyama et al. 2013; Matusek et al. 2014; Valentine et al. 2014; Jacomin et al. 2016). Similarly as with the MS1096-Gal4 driver, the en-Gal4 directed expression of the *Chmp1* silencing transgenes resulted in observable wing defects at the adult stage that were fully rescued by the wild-type human CHMP1B but only partially rescued by the ubiquitination-defective mutant (Fig. R5C).

Our results therefore demonstrate the requirement of CHMP1B in wing development, including the restriction of Notch activation domain to the wing margin, and show the importance of the modification of CHMP1B by ubiquitin linkage for its function in *Drosophila* suggesting the evolutionary conservation of an ubiquitination-based regulation of CHMP1 proteins.

7. General Conclusion

The results presented in my thesis allow to draw a model for CHMP1B activation and polymerization at the endosomal membranes under the control of UBPY as shown in the graphical abstract below.



Legend

CHMP1B activation is dynamically regulated by ubiquitination and the ESCRT-associated ubiquitin specific protease UBPY (synonym USP8). CHMP1B is rapidly and transiently ubiquitinated in its helical core in response to EGF inducing or stabilizing an open conformation. Ubiquitinated monomers or dimers of CHMP1B are in an open-inactive conformation where ubiquitin moieties block their polymerization. These ubiquitinated dimers of CHMP1B accumulate on membranes within minutes following EGF stimulation and subsequent deubiquitination by UBPY triggers or allows CHMP1B oligomerization.

In this model, we notably hypothesize that the linkage of an ubiquitin molecule in the core helix of CHMP1B primes the protein for polymerization by stabilizing its open conformation, while its subsequent oligomerization requires ubiquitin hydrolysis by UBPY at the endosomal membranes. Thus ubiquitination serves as a dynamic checkpoint for CHMP1B activation and oligomerization.

The demonstration that CHMP1B ubiquitination is dynamically regulated by EGF or inflammatory cytokines, and is required for a fully functional protein in EGFR trafficking and wing formation in *Drosophila* corroborate the physiological relevance of this post-translational modification.

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VI. Annexes

ANNEX I: The Genetics of the Drosophila

A. *Drosophila Melanogaster* as a Model Organism

Most of what we know about the biology of cells and tissues comes from studies using model organisms such as mice or the fruit fly *Drosophila melanogaster*.

Drosophila melanogaster has been used productively as a model organism for over a century because is easy and inexpensive to raise in laboratory conditions, have a very shorter life cycle, produce large numbers of externally laid embryos and can be genetically modified in numerous ways without presenting ethical obstacles. That is why most of the conserved fundamental biological mechanisms and pathways that control development and survival have been discovered in *Drosophila*.

Numerous genetic modification can be performed quite easily in the *Drosophila*: gene expression or inactivation in specific tissues, specific periods of development or even in only certain cells of the insect. The assortment of transgenic strains is organized in Flybase, and are available from the labs that generated them or from stock centers.

B. Generation of UAS-Gal4 *Drosophila* Lines Using P-elements

One of the most powerful genetic manipulation in *Drosophila* is the use of the UAS-Gal4 derived from the yeast *Saccharomyces cerevisiae*. The engineering of the UAS-Gal4 system into the *Drosophila* system was developed by Brand and Perrimon using the transposable P-elements (Brand and Perrimon 1993).

P-elements can be autonomous or non-autonomous**.** The autonomous P-elements contains inversed repeated sequence in 3' and 5' and the four exons coding for the protein Transposase that catalyzes the genomic mobility of the element. The non-autonomous P-elements don not contain the transposase and it has to be provided externally.

First of all the transgenic DNA has to be cloned into a vector plasmid or viral which will transfer it to an intra-chromosomal site in a germ line cell to allow for stable and hereditary transformation. The vector used for this transfer is the P-element. *Drosophila* embryos are injected with two different P-element containing plasmids: one carries the transgene and the other the source of transposase. The transgene consists of the DNA

sequence of interest and a transgenesis phenotypic marker, typically the w + allele of the white gene. The transposase is produced in the embryo and can then catalyze the insertion into the chromosomal DNA of the transgenic DNA contained in the first P-element plasmid by performing a cut at the feet. The transferred gene is stable (non-reworked) and is stably transmitted to subsequent generations if the insertion was made in the nucleus of a gem cell.

For the generation of the UAS-Gal4 system, the first P-element contains a promoter associated with a gene encoding the yeast transcription factor Gal4. The Second element P carries the transgene of interest downstream of a promoter containing the UAS sequences recognized by the Gal4 factor. This target sequence is silent in the absence of Gal4. The crossing of a driver line (containing the element P {Gal4}) with a target line (containing the element P {UAS-sequence of interest}) allows the expression of this sequence of interest. Depending on the nature of the Gal4 promoter, the expression of a transgene can be controlled to induce in a specific tissue or at a specific time Development. There are also heat shock promoters (hsp) which induce the transcription of the factor Gal4 in the event of heat shock (Fig. A1).

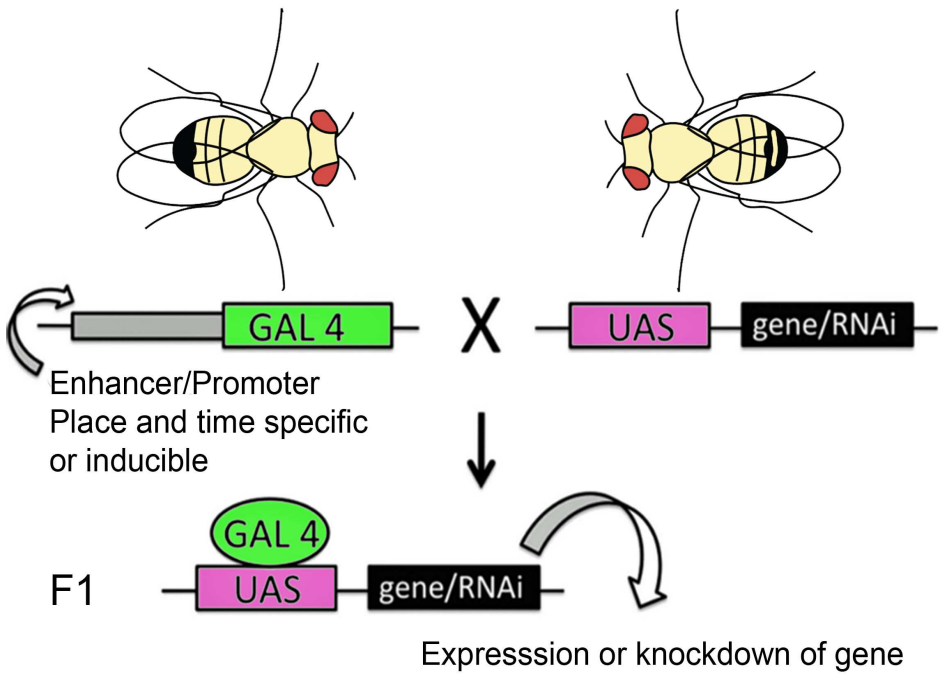

Figure A1: Gal4-UAS system in Drosophila: The two parental lines of Drosophila are represented. **Left:** DRIVER line expressing the transcription factor Gal4 under the control of a tissue-specific or inducible promoter. **Right:** TARGET line possessing the sequence coding for the transgene or the RNAi (repeated inverse sequence of the targeted DUBs mRNA) under the control of a UAS promoter. (The RNAi is therefore not expressed in the target line which is wild phenotype wild). The descendants in the F1 resulting from the crossing combines in its genome the expression of the factors Gal4 allowing the expression of the transgenes or the RNAi enhancer/promoter specific manner.

C. The FLp-FRT System in Drosophila to Generate Cell Clones

The FLP-FRT system is similar involves using flipase (FLP) recombinase, derived from the yeast *Saccharomyces cerevisiae*. FLP recognizes a pair of FLP recombinase target (FRT) sequences that flank a genomic region of interest.

In the system used in this study the FLP recombinase is under the control of a heat shock promoter (hsp). In the same genome two FRT sites flank a sequence to eliminate between, a ubiquitous promoter and a transgenic or reporter sequence. Under a heat-shock, the flipase induces the recombination of the FRT sites in the somatic cells leading to the loss of the gene contained between these FRT sequences and the ubiquitous promoter is then able to induce transcription of the transgenic sequence of interest. The sequence of interest can be the Gal4 transcription factor and this can be combined with the introduction of UAS-gen of interest sequences allowing the clonal analysis of mutant generated by the UAS-Gal4 system.

This system was used during my thesis to generate clones co-expressing the GFP-nls, GFP-Rab7 or GFP-Lamp1 and RNAi for dCHMP1. In this set up, mutant cells are surrounded by wild type tissue, and cell autonomous effects of the RNAi can be observed.

CRIPSR/CAs9 (or in discussion) although it has not been used in this study, this powerful system allowing endogenous gene mutation or editing works super efficiently in Drops hila :

D. The Engrailed-Gal4 Notch Activity Reporter Line

The genotype of the line used in study to follow the activation of the Notch pathway in the larval wing tissue of the Drosophila is as follow:

w^{1118}; en-Gal4, UAS-Myr-RFP, NRE-EGFP/+

In the second chromosome has been engineered the introduction of the Gal4 gene under control of the Engrailed promoter, which is expressed in the posterior compartment cells of the wing in the L3; the reporter gene Myr-RFP which is a membrane protein attached to the red-fluorescent-protein under the UAS control to follow which cells are expressing Gal4; and the reporter gene NRE-EGFP which drives the expression of the green-fluorescent protein EGFP under the control of the Notch Responsive Elements (this elements drive expression of the downstream gene when the Notch pathway is activated). Therefore, when you cross this line with the fly lines containing CHMP1B transgenic constructs or RNAi under the control of UAS sequences in the third chromosome, in the offspring, it is possible to follow the effect of the expression of this CHMP1 transgenes on the activation of the Notch signaling pathway, and compare it with the wild type tissue of the anterior compartment of the wing into the same individual and organ.

ANNEX II: Drosophila Wing Development

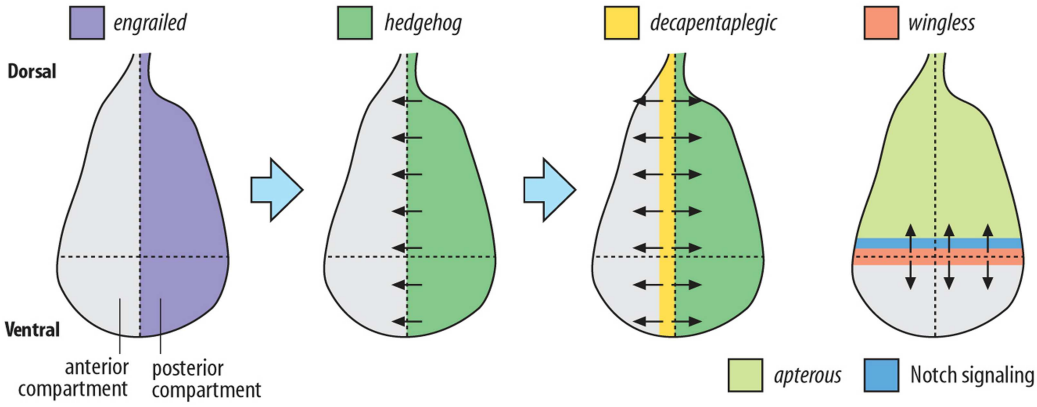

Figure A2: Signaling events during Drosophila WID development. To simplify the disc is structured into two axes. The anterior/posterior axis is structured by the segment polarity genes engrailed and hedgehog on either side of a stripe expressing decapentaplegic. The dorso-ventral axis is structured by Vn-EGFR (see Figure 32) and apterous. Wingless serves as organizing center and to structure the sensory hairs at the edges of the wing (picture modified from Dr. Brian E. Staveley)

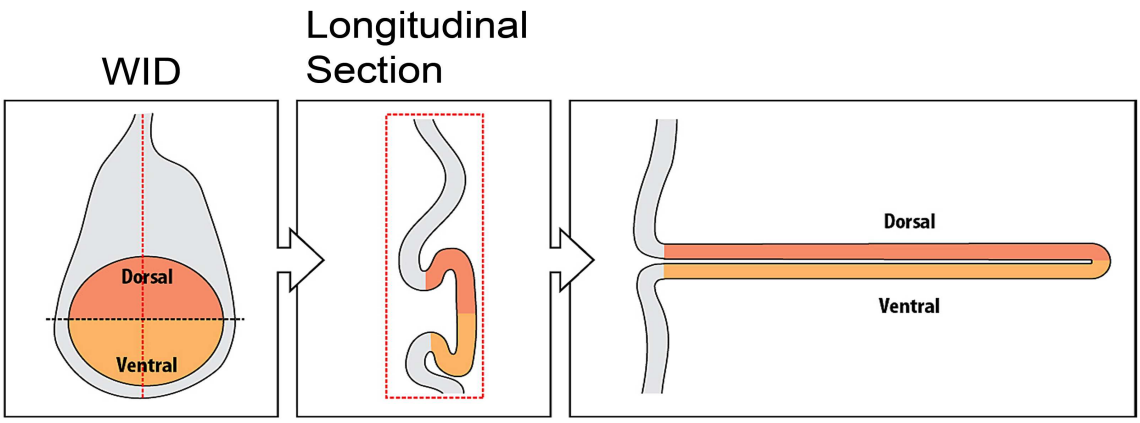

Figure A3: Formation of the wing pouch: The wing pouch is formed from the embryonic ectoderm by an invagination at the intersection of a dorsal/ventral stripe of Wingless with an anterior-posterior stripe of Decapentaplegic (see Figure A2) (picture modified from Dr. Brian E. Staveley)

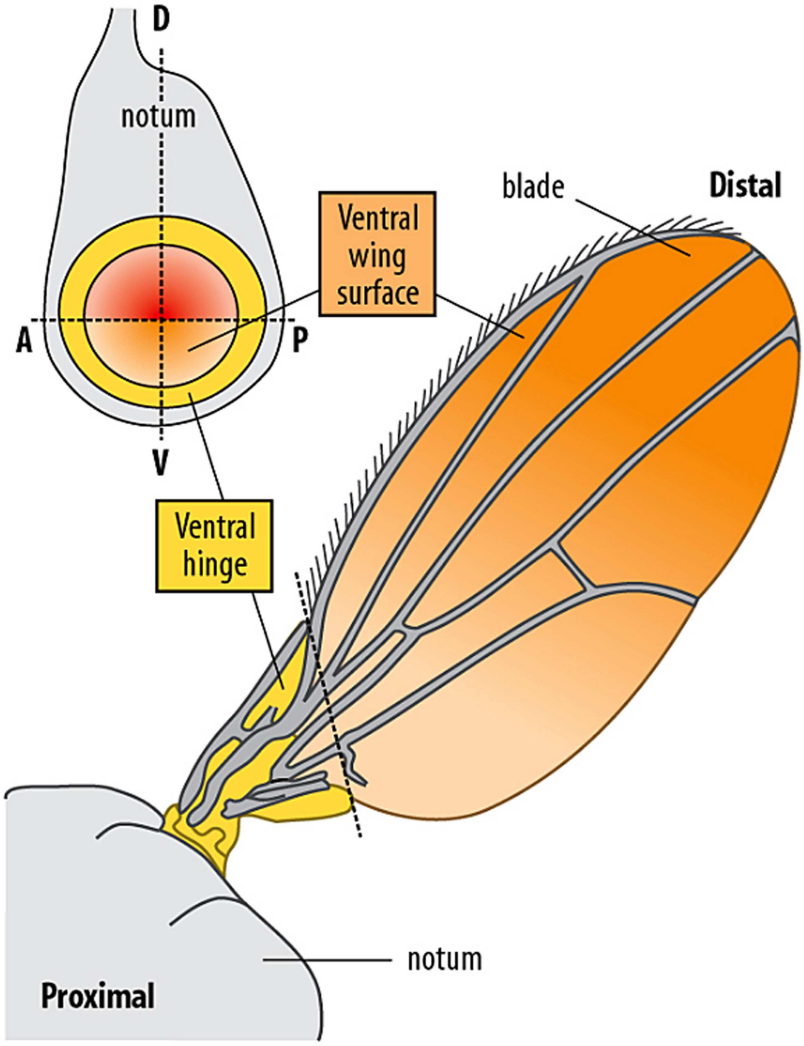

Figure A4: Equivalence between WID larval structures and adult wing structures

ANNEX III: CHMP1B Protein

hCHMP1B Protein (From UniProt).

{Q7LBR1} 1-199aa

```
        10         20         30         40         50         60         70
MSNMEKHLFN LKFAAKELSR SAKKCDKEEK AEKAKIKKAI QKGNMEVARI HAENAIRQKN QAVNFLRMSA
        80         90        100        110        120        130
RVDAVAARVQ TAVTMGKVTK SMAGVVKSMD ATLKTMNLEK ISALMDKFEH QFETLDVQTQ
       140        150        160        170        180        190
QMEDTMSSTT TLTTPQNQVD MLLQEMADEA GLDLNMELPQ GQTGSVGTSV ASAEQDELSQ
      199
RLARLRDQV
```

αHelix1: 3-42; **αHelix2:** 44-104; **αHelix3:** 108-126; **αHelix4:** 128-139; **αHelix5:** 146-160; **αHelix6:** 177-195 (Bajorek et al. 2009).

MIT domain. Coiled Coil Domains (UniProt.org)

Ubiquitinated Predicted Sites from Udeshi ND 2013 proteomic analysis (nextprot.org): Lysines residues: 12, 42, 87, 104, 110

Table 3: CHMP1B interactions (UbiProt.org)

Residues	Interaction
132-156	IST1
174-199	Spastin
180-199	Vta1
180-196	Vps4A, MITD1,STAMBP
183-199	Vps4B

ANNEX IV: Additional Results

1. Conservation of K87 and K90

```
Arabidopsis t       MGNTDKLMNQIFELKFTSKSLQRQARKCEKEERSEKLKVKKAIEKGNMDGARIYAENAIR 60
Homo sapiens        ---MSNMEKHLFNLKFAAKELSRSAKKCDKEEKAEKAKIKKAIQKGNMEVARIHAENAIR 57
Drosophila m        -MSTSSMEKHLFNLKFAVKELERNSKKCEKEEKLEKAKAKKAIQKGNMDVARIHAENAIR 59

Arabidopsis t       KRSEQMNYLRLSSRLDAVVARLDTQAKMATITKSMTNIVKSLESSLTTGNLQKMSETMDS 120
Homo sapiens        QKNQAVNFLRMSARVDAVAARVQTAVTMGKVTKSMAGVVKSMDATLKTMNLEKISAIMDK 117
Drosophila m        QKNQAVNYLRMSARVDAVASRVQSALTTRKVTGSMAGVVKAMDAAMKGMNLEKISSIMEK 119

Arabidopsis t       FEKQFVNMEVQAEFMDNAMAGSTSLSTPEGEVNSLMQQVADDYGLEVSVGLPQPAGH-AI 179
Homo sapiens        FEHQFETLDVQTQQMEDTMSSTTTLTTPQNQVDMLLQEMADEAGLDLNMELPQGQTG-SV 176
Drosophila m        FESQFEDLDVQSSVMEGTMSDTVTTSVPQGDVDNLLQQVADEAGLELNMELPSGVQSQSV 179

Arabidopsis t       PTKTEEKVEEDDLTRRLAELKARG       203
Homo sapiens        GT-SVASAEQDELSQRLARLRDQV       199
Drosophila m        GASTAVSQEQDELTQRLARLRQAE       203
```

Figure A5: Clustal analysis of the protect sequence of Vps46.1 of *Arabidopsis Thaliana*, Chmp1b of *Homo sapiens* and Chmp1 *of Drosophila melanogaster*.

2. Western blot analysis of other CHMPs

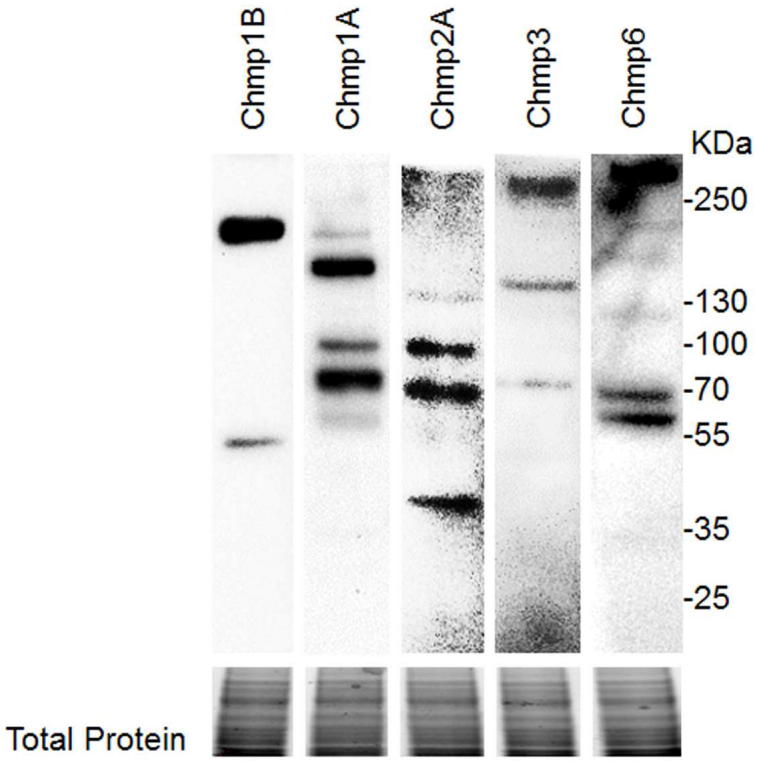

Figure A6: WCL of non-treated HEK293T cells were analyzed by western blot using anti-CHMP1B, CHMP1A, CHMP2, CHMP3 and CHMP6 antibodies.

3. No co-localization of CHMP1B with Rab7 positive compartment in Drosophila fat body cells

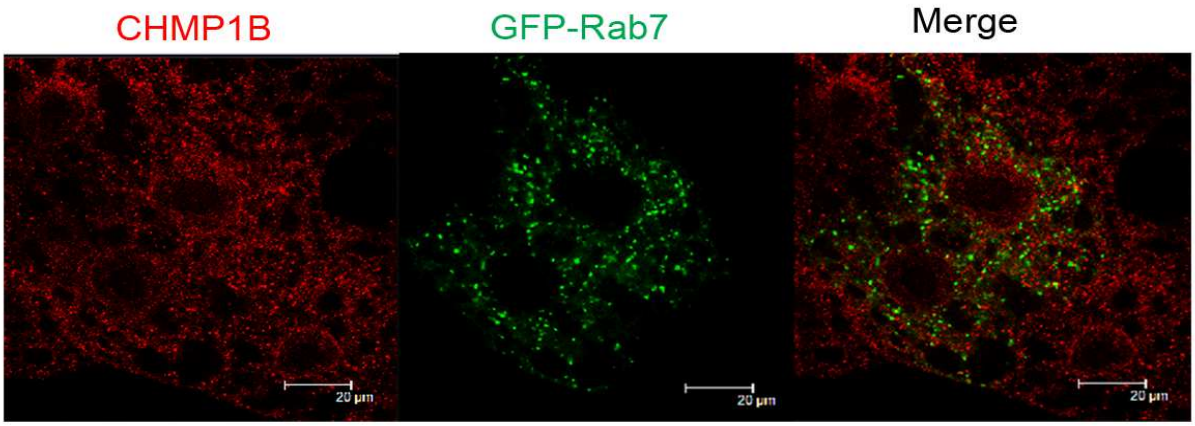

Figure A7: larval fat body cell clones expressing the fusion protein GFP-Rab7 stained with anti-CHMP1B antibody.

4. EGFR turnover in steady-state and degradation upon stimulation with EGF is regulated by CHMP1B

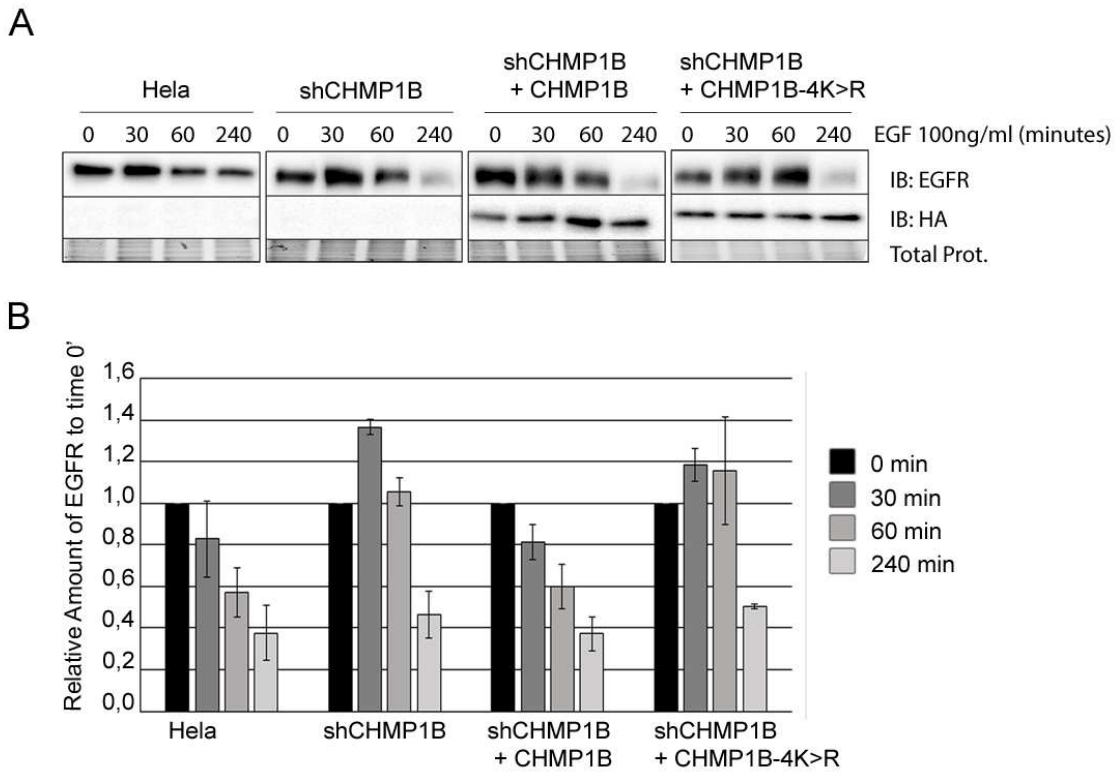

Figure A8: Analysis of lysates from HeLa cells expressing or not a shRNA-CHMP1B against 3'UTR region (sh94) and transfected with HA-empty, HA-CHMP1B wild type or HA-CHMP1B-4K>RR. Cells were subjected to serum starvation prior to stimulation by EGF and lysed at the indicated time points prior to analysis. Proteins were separated by SDS-PAGE and revealed by IB using either anti-EGFR or anti-HA antibodies.

5. CHMP1BΔMIM dominant negative effect in the Drosophila eye

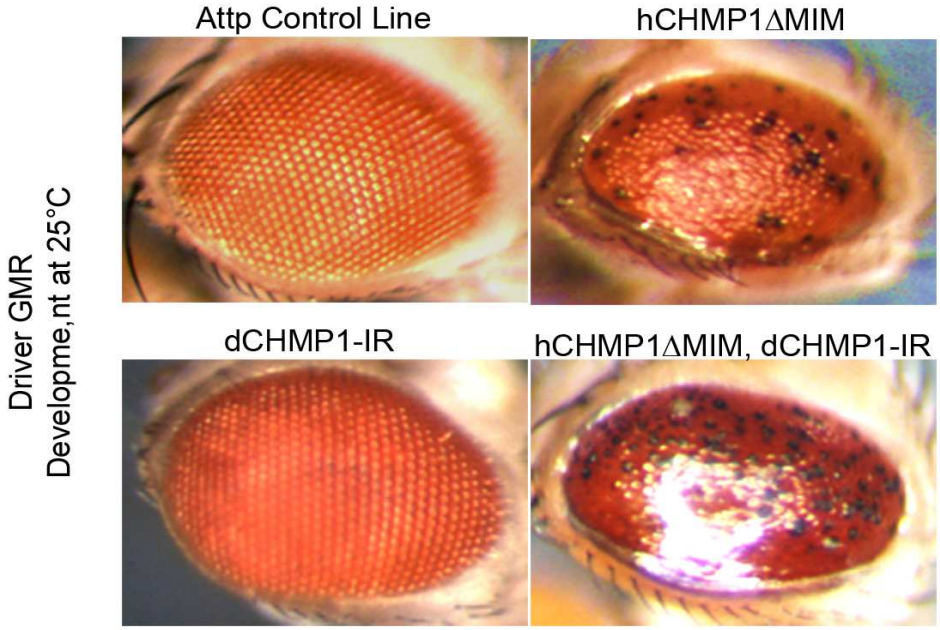

Figure A9: Picture of the eye of 3-5 day-old males flies raised at 25°C and expressing indicated transgenes under the control of the GMRGal4 driver which is expressed mostly in the eye. Genotypes are as follows: **Ctrl:** MS1096/+; UAS-attp40. ***dCHMP1-IR***: MS1096/+; *UAS-lacZ*/+;UAS-*CHMP1-IR*/+. **hCHMP1B-ΔMIM:** MS1096/+; *UAS*- hCHMP1B- ΔMIM/+; *UAS-birA* **hCHMP1B- ΔMIM + *CHMP1-IR*:** MS1096/+; *UAS-dCHMP1-IR*/+*; UAS*-hCHMP1B- ΔMIM/+.

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Résumé

Ma thèse porte sur la biogenèse de vésicules au sein d'un compartiment intracellulaire spécialisé dans le recyclage ou la destruction de récepteurs membranaires. Ce tri des récepteurs, suite à leur internalisation par endocytose depuis la surface vers l'intérieur de la cellule, régule directement la réponse à des signaux extracellulaires au cours du développement et de la vie adulte. Les résultats présentés démontrent l'existence et le rôle de la liaison d'une molécule d'ubiquitine dans l'activation d'une protéine d'endocytose (CHMP1B) en réponse à un facteur de croissance et de différenciation cellulaire (l'EGF) ou de cytokines inflammatoires (TNFa, IL1). En outre, ils mettent en évidence le rôle d'une protéase (UBPY syn. USP8) dans l'hydrolyse de cette ubiquitine et la polymérisation de CHMP1B à la membrane. Ce nouveau concept permet de mieux comprendre les mécanismes de déformation et de scission des membranes intracellulaires dans des processus physiologiques ou pathologiques.

Abstract

My thesis concerns the biogenesis of vesicles within an intracellular compartment specialized in the recycling or destruction of membrane receptors. This sorting of the receptors following their internalization by endocytosis from the surface towards the inside of the cell directly regulates the response to extracellular signals during development and adult life. The results presented demonstrate the existence and role of binding of a ubiquitin molecule in the activation of the endocytic protein (CHMP1B) in response to cell growth and differentiation factor (EGF) or to inflammatory cytokines (TNFa, IL1). In addition, they show the role of a protease (UBPY syn. USP8) in the hydrolysis of this ubiquitin and the polymerization of CHMP1B at the membrane. This new concept allows to better understand the mechanisms of deformation and scission of intracellular membranes in physiological or pathological processes.