Study of cellular responses to membrane damages

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THESE

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This part will be completed for the final manuscript.

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Abbreviations

2 D System: 2-dimension system

A(PKA)

AAA ATPase VPS4: ATPase Associated with varius cellular Activities

AC: After Christ

ALFY: Autophagy-Linked FYVE

AMSH: Associated Molecular with Sh3 domain of STAM

Arf6: ADP-ribosylation factor

ASM: Acid SphingoMyelinase

Atg: Autophagy-related

ATP: Adenosine TriPhosphate

ATP6VOA2: subunit of the vacuolar ATPase

BAR: Bin1/Amphiphysin/Rvs167

BFA: Brefeldin A

C2C12 cells: is a mouse myoblast cell line

Ca^{2+}: Calcium Ion

CA3: Histological division of the hippocampus

cAMP: cyclic Adenosine MonoPhosphate

CCCP: carbonyl cyanide 3-chlorophenylhydrazone

CCP: calcium phosphate precipitate

CD2AP: CD2-associated protein

CEP55: CEntrosomal Protein 55kDa

Cer: ceramides

CHMP: CHarged Multivesicular body Proteins

CHMP2B^{intron5}

CHOL: colesterol

CL: Cardiolipin

CMA: Chaperone- Mediated Autophagy

COG: Conserved Oligomeric Golgi Complex

COP- II: COat Protein II

COP-I: COat Protein I

COS-7 cells: CV-1 (simian) in Origin, and carrying the SV40 genetic material

CREB: cAMP responsive element binding protein Cvt: Cytoplasm to vacuole targeting

CXCR4: chemokine (C-X-C motif) receptor

Cy3, Cy5: Cyanine 3, Cyanine 5

DAB: DiAminoBenzidine

DAG: DiAcylGlycerol

DAG: DiAcylGlycerol

Did2yeast/CHMP1mammals

DMEM: Dulbecco's Modified Eagle Medium

DNA: DeoxyriboNucleic Acid

DOPS: 1,2-Dioleoyl-sn-glycero-3-phosphoserine (type of Phosphatidylserine)

DUB: DeUBiquitinating enzyme

E1, E2, E3, E4: Enzyme 1, Enzyme 2, Enzyme 3, Enzyme 4

EAP45: ELL-Associated Protein

EGFP: enhanced green fluorescent protein

â¨LC3: microtubule-associated protein 1 light chain 3 beta

EGFR: Epidermal Growth Factor Receptor

EDM: Electron Dense Material EM: electron microscopy

ENaC: Epithelial Na^+ channel

EPS-15: Epidermal growth factor receptor Pathway Substrate 15

Epsin1: EPS-15-INteracting protein 1

ER: Endoplasmic Reticulum

ESCRT: Endosomal Sorting Complex Required For Transport

FACS: fluorescence activated cell sorting

FBS: Foetal Bovine Serum

FGF: Fibroblast Growth Factor

FGF2: Fibroblast Growth Factor 2

FIP: Family Interacting Protein

FRAP: Fluorescence Recovery After Photobleaching

FSGS: Focal Segmental GlomeruSclerosis

FVVE: Fab1p, YOTP, Vac1p, EEA1

G- coupled receptors: Guanine nucleotide coupled receptors

G-proteins: Guanine nucleotide binding protein

GABARAP: γ-aminobutyric-acid-typeA

(GABAA)-receptor-associated protein
GABARAPL: γ-aminobutyric-acid-typeA
(GABAA)-receptor-associated like protein
Gag: Group-specific AntiGen
GalCer: GalastosyICeramide
GalT: β-1,4-galactosyltransferase 1
GalT: β-1,4-galactosyltransferase 1
GAT: GGAs And TOM
GATE 16: Golgi-associated ATPase enhancer of 16kDa
GGAs: Golgi-associated, γ-Adaptin ear containing, ARF binding protein
GlcCer: GlucosyICeramide
GLUE: GRAM-like ubiquitin-binding in EAP45
GM130: Golgi Matrix protein 130kDa
GPHR: G Protein-Coupled Receptor
GSL: GlycoSphingoLipids
GTP: Guanosine TriPhosphate
GUV: Giant Unilamellar Vesicle
HEK 293T: Human Embryonic Kidney cell line
HII: Hexagonal - II
HIV-1: Human Immunodeficiency Virus 1
HRP: Horseradish peroxidase
HRS: Hepatocyte growth factor-Regulated tyrosine kinase Substrate
Hsc70: Heat Shock Cognate 70 k Da
Hse1: Heparanase 1
IF: ImmunoFluorescence
ILV: IntraLuminal Vesicle
ISL: Inositol SphingoLipid
IST1: Increased Sodium Tolerance 1
KD: Knock-Down
kDa: kiloDalton
KO: Knock-Out
LAMP: Lysosomal Associated Membrane Protein
LBPA: LysoBisPhosphatidic Acid
LC3: Light-Chain 3 (also known as microtubuke-associated protein 1 light chain 3)
LIP5: LYST-Interacting Protein 5
LIR: LC3-Interacting Region

LPA: LysoPhosphatic Acid
LPV: LC3 positive vacuole
Lys48: Lysine in position 48 of the Ubiquitin protein
Lys63: Lysine in position 63 of the Ubiquitin protein
ManII: α-mannosidase II
MEF: Mouse Embryonic Fibroblast
MG53: MitsuGumin-53
MHC-I: Major Histocompatibility Complex I
MIM: MIT Interacting motif
MIT: Microtubule Intercating and Transport
MLV: Murine Leukemia Virus
MVB: MultiVesicular Body
NAC: N-acetyl cysteine
NBR1: neighbor of BRCA1 gene 1
NDP52: Nuclear dot protein 52kDa
Nedd4 like: Neural precursor cell Expressed, Developmentally Down-regulated 4 -like
NZF: Npl4 Zinc Finger
PA: Phosphatic Acid
PA: Phosphatic-Acid
PB1: Phox and Bem 1pdomain
PBS: Phosphate Buffer Saline
PC: PhosphatidylCholine
PCD- Programmed Cell Death
PCR: Polymerase Chain Reaction
PDx: Pancreatic and Duodenal homeobox
PE: PhosphatidylEthanolamine
PFT: Pore-Forming Toxin
PG: PhosphatidylGlycerol
PH: Pleckstrin Homology
PI: Phosphatidyl Inositol (in Chapter 1)
PI: Propidium Iodide (in Chapters 2, 3, 4 and Results1)
PI 3-kinase: PhosphoInositide 3-kinase
PI(3,4,5)P3: PdtIns(3,4,5)P2 or Phosphatidyl Inositol-(4,5)-TriPhosphate
PI(3,4)P2: PdtIns(3,4)P2 or Phosphatidyl Inositol-(3,4)-Phosphate
PI(3,5)P2: PdtIns(3,5)P2 or Phosphatidyl

Inositol-(3,5)-BiPhosphate

PI(4,5)P2: PdtIns(4,5)P2 or Phosphatidyl Inositol-(4,5)-BiPhosphate

PI3K: Phosphatidynositol-3-Kinase

PI3K-III: III PhosphoIonositide3-Kinase

PI3P: PdtIns3P or Phosphatidyl Inositol-3-Phosphate

PI4P: PdtIns4P or Phosphatidyl Inositol-4-Phosphate

PKC: proteÃn-Kinase-C

PLA2: PhophoLipase A2

PMN: Piecemeal Microautophagy of the Nucleus

PrP-Sc: Prion Protein Scrapie-associated

PrP: Prion Protein

PRR: PRolin Rich

PS: PhosphatidylSerine

PS: PhosphatidylSerine

PTPN23: Protein Tyrosine Phosphatase, Non-receptor type 23

PTRF: Polymerase Transcriptase Release Factor

RNF 185: RiNgFinger protein

RT: Room Temperature

S1P: Sphingosine1

SDS: sodium dodecyl sulfate

SEC23B

SI: International System of Units

siRNA: small interfering RiboNucleic Acid

SLO: Streptolysin-O

SM: SphingoMyelin

SMURF1

SNARE: Soluble N-ethylmaleimide-sensitive-factor Attachment protein REceptor

SOD1: Superoxide dismutase- type 1

SOPC: 1-Stearoyl-2-oleoyl-sn-glycero-3-phosphocholine (type of Phosphatidylcholine)

Sph: Sphingosine

SPM: SPhingoMyeline

SQSTM1 (or p62): SeQueSTosoMe1

STAM: Signal Transducing Adaptor Molecule

TEM: transmission electron microscope

TG: TriacyGlycerol

TGN: Trans-Golgi Network

WT: Wild-Type

TOMs: Target Of Myb

TRAP III Complex: TRAnsport protein Particle

TSG101: Tumor Susceptibility Gene 101

UBA domain: Ubiquitin-associated domain

UBD: Ubiquitin Binding Domain

UBPY: UBiquitin isoPeptidase Y

UEV: Ubiquitin E2 Variant

UIM: Ubiquitin-Intercating Motif

ULK(ATg1 in yeast)

UV-light: Ultra Violet light

VHS: Vps27 HRS STAM

VLP: Virus-Like Particle

VPS: Vacuolar Sorting Proteins

VPS60yeast/CHMP5mammals

Vta1: Vps20-Associated 1

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General introduction

Abstract

The integrity of cell membranes is essential for their survival and for the proper functioning of the cell. However, these membranes are constantly exposed to physical, chemical and biological threats that can damage them. The first part of this research concerns the study of plasma membrane repair following exposure to physical assaults (lasers or micro-needles), and chemical (amphipathic pores forming molecules) or biochemical toxins. We describe a new mechanism for plasma membrane repair that involves the complex III of the ESCRT (endosomal sorting complex required for transport) machinery, whose functions were restricted to the biogenesis of intraluminal vesicles of multivesicular endosomes, in cytokinesis and budding of viruses such as HIV. Our observations suggest that the ESCRT machinery may be involved in removing damaged portions of the plasma membrane by budding. We believe that our results represent a valuable contribution to the field of ESCRTs. We also propose that our system, which is specific and controlled, could be used to further understand the mechanisms of the ESCRT machinery and further explain their implications, not only in membrane repair, but also in the other cellular processes where ESCRTs function.

The second part of this study focuses on the cellular response to physical (laser) damage or chemical (by chemical cross-linking) damage of trafficking organelles such as endosomes and the Golgi apparatus. We demonstrate the involvement of the mechanisms of autophagy in response to endosome and Golgi apparatus damage. This response includes the rapid recruitment of the protein LC3 which is the main protein detectable on the membranes of mature autophagosomes. We have also shown the involvement of ubiquitination and the rapid recruitment of proteins p62 and NBR1, which are proteins that can bind both ubiquitin and LC3. The observed mechanism has much in common with other mechanisms of selective autophagy but nevertheless reveals peculiarities such as the direct recruitment of LC3 on the membranes of damaged Golgi apparatus.

Thus, our research includes the study of two cellular mechanisms of response to membrane damage that highlight the existence of mechanisms for systematic monitoring of homeostasis of organelles and the plasma membrane. These mechanisms are adapted to the specific nature of the damaged membranes. First, we show that damage to certain trafficking organelles (endosomes and the Golgi apparatus) triggers the response machinery of autophagy whilst damage to the plasma membrane induces a new repair mechanism involving the machinery of ESCRTs.

Abstract

L'intégrité des membranes cellulaires est essentielle à la survie et au bon fonctionnement de la cellule. Or, ces membranes sont constamment exposées à des agents physiques, chimiques ou biologiques susceptibles de les endommager.

La première partie de cette étude porte sur la réparation de la membrane plasmique perforée suite à l'exposition à des contraintes physiques (lasers ou micro-aiguilles), chimiques (molécules amphipatiques formant des pores dans les membranes) ou biochimiques (toxines). Nous décrivons un nouveau mécanisme de réparation de la membrane plasmique qui met en jeu le complexe III de la machinerie des ESCRT (endosomal sorting complex required for transport), dont les fonctions étaient limitées jusqu'à présent à la biogenèse des vésicules intraluminales des endosomes multivesiculaires, à la cytocinèse et au bourgeonnement de virus tels que le VIH. Nos observations suggèrent que la machinerie des ESCRTs serait impliquée dans l'élimination de portions endommagées de membrane plasmique par bourgeonnement. Cette observation suggère par ailleurs de nouvelles hypothèses permettant d'unifier les autres mécanismes où les ESCRTs sont impliquées. Nos résultats portent une information novatrice dans le domaine des ESCRTs. Notre systéme est un systéme simple, sans dangers et contrôlé. Nous proposons que notre système peux constituer un outil de choix pour comprendre plus en détail le mode de fonctionnement de la machinerie des ESCRTs. Il pourrait nous permettre de comprendre plus en détail le rôle des ESCRTs, non seulement dans la réparation membranaire, mais aussi dans les autres functions cellulaires où les ESCRTs sont impliquées.

La deuxième partie de cette étude porte sur la réponse cellulaire à l'endommagement physique (laser) ou chimique (par pontage chimique) d'organites de trafic tels que les endosomes ou l'appareil de Golgi. Nous montrons l'implication des mécanismes d'autophagie dans la réponse à l'endommagement des endosomes et de l'appareil de Golgi. Cette réponse comprend un recrutement rapide de la protéine LC3 qui est une des seules protéines détectables sur les membranes des autophagosomes matures. Nous avons montré également l'implication d'une ubiquitination rapide ainsi que du recrutement des protéines p62 et NBR1, qui sont des protéines capables de lier à la fois l'ubiquitine et la protéine LC3. Le mécanisme observé présente de nombreux points communs avec d'autres mécanismes d'autophagie sélective mais révèle néanmoins des particularités comme le recrutement direct de LC3 sur les membranes de l'appareil de Golgi endommagé. Notre étude comprend donc l'étude de deux mécanismes cellulaires de réponse aux endommagements de membrane qui mettent en évidence l'existence de mécanismes de surveillance systématique de l'homéostasie des organelles et de la membrane plasmique. Ces mécanismes sont adaptés à la nature des membranes endommagées. D'une part, nous montrons que l'endommagement de certains organites de trafic (les endosomes et l'appareil de Golgi) déclenche une réponse de la machinerie de l'autophagie alors que d'autres part un endommagement de la membrane plasmique induit un nouveau mécanisme de réparation impliquant la machinerie des ESCRTs.

Part I

Repairing the wounded plasma membrane

The ESCRT proteins: Scaffolds for negative curvature and membrane fusion and fission

As we saw above, various mechanisms can lead to membrane deformation and membrane fusion/fission. Nevertheless, in the case of budding vesicles, most mechanisms described deform the membrane in the direction of the cytosol. The release of these vesicles occur by constriction of the neck driven by machineries that surround it. Such vesicles are subsequently released into the cytoplasm after the severing of the neck. Up to now, only the ESCRT proteins have been described to allow the formation and release of vesicles in the opposite direction, in the extracellular medium (virus budding) or into the lumen of organelles (the biogenesis of Intra Luminal Vesicles, ILVs, in the lumen of MVBs). The deformation of the membrane driven by ESCRTs involves their binding to the membrane leaflet that will be found inside the vesicle. Similarly, they have been proposed recently to be involved in the processing of the cytoplasmic bridge during cytokinesis that presents the same topology in terms of membrane fusion.

Therefore, ESCRTs are crucial for cell division and correct trafficking of certain cargos, and in addition, they play a major role during virus infection that need to be understood for the fight against pathogens.

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1.1   The ESCRT complexes

The ESCRT proteins were first defined as the class E subset of the Vacuolar Sorting Proteins (VPS). Interfering with the activity of any of these proteins in yeast would cause missorting of cargo (normally heading to the vacuole) which then accumulates in abnormal multicisternal compartments called class E compartments [Raymond 1992]. Further studies showed that class E Vps Proteins can assemble into three complexes: ESCRT-I [Katzmann 2001], ESCRT-II [Babst 2002b] and ESCRT-III [Babst 2002a]. A member of the fourth ESCRT complex was first identified: the AAA ATPase VPS4 which is also necessary for cargo delivery into the vacuole [Babst 1997]. Later on, a fifth complex acting upstreams of the ESCRT-I complex was identified [Babst 2002b, Bache 2003b, Bache 2003a, Bilodeau 2002, Katzmann 2003, Williams 2007]. This complex is termed ESCRT-0. Very recent *in vitro* studies have confirmed the order of recruitment of ESCRT complexes to : 1) ESCRT-0, 2) ESCRT-I, 3) ESCRT-II, 4) ESCRT-III, 5) ESCRT disassembly [Saksena 2009b, Wollert 2009, Wollert 2010]. The interactions between the different subcomplexes and between the subunits within a subcomplex are presented in Figure 2.1.
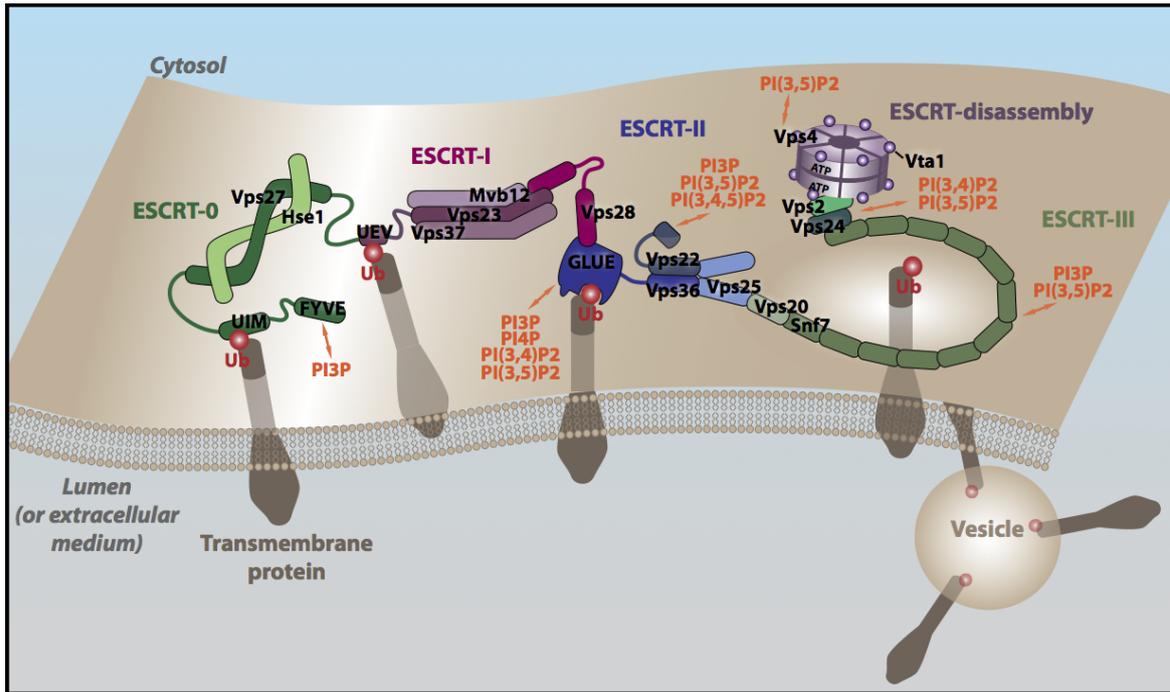
FIGURE 1.1 – **The ESCRT complexes: interactions between the subunits of each ESCRT sub-complex and interactions between sub-complexes.**

The ESCRT-0 complex is composed of $Vps27_{yeast}/HRS_{mammals}$ and $Hse1_{yeast}/STAM1\text{-}2_{mammals}$(Stoechiometry 1:1). The ESCRT-I complex is composed of $MVB12_{yeast}/MVB12A \& MVBB_{mammals}$, Vps23, Vps37 and Vps28 (Stoechiometry 1:1:1:1). ESCRT-I is recruited *via* the interaction between its subunit Vps23 and Vps27/HRS (ESCRT-0). The ESCRT-II complex is composed of Vps22, Vps36 and Vps25 (Stoechiometry 1:1:1). It is recruited thanks to the interaction between Vps28 (ESCRT-I) and Vps36(ESCRT-II). The ESCRT-III complex is composed of $Vps20_{yeast}/CHMP6_{mammals}$, $Snf7(or\ Vps32)_{yeast}/CHMP4A$, B and $C_{mammals}$, $Vps24_{yeast}/CHMP3_{mammals}$ and $Vps2_{yeast}/CHMP2A$ and $B_{mammals}$ (Stoechiometry not known). ESCRT-III is recruited *via* the interaction between its subunit Vps20/CHMP6 and Vps25 (ESCRT-II). The subunits touching each-other in the scheme, interact with each-other. Orange-arrows and orange letters represent the interactions between ESCRT subunits and lipids. Based on [Teis 2009].

Many of the yeast ESCRTsubunits have one or several mammalian homologs. The role of these homologs is poorly understood but it is likely that for many of them, the existence of several homologs implies specialization for different functions in the cell. In some other cases, these homologs may be involved in the regulation of each other. This has been shown in cytokinesis for the mammalian ESCRT-III proteins CHMP4B and C which have opposite effects on cytokinesis speed. These homologs may be crucial for the regulation of events like cell division and they deserve careful study.

1.1.1 The ESCRT-0 complex
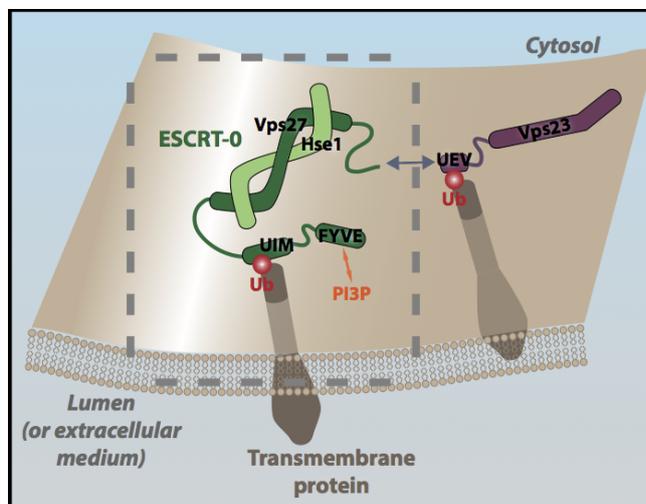
FIGURE 1.2 – **The ESCRT-0 complex**
The ESCRT-0 subcomplex corresponds to the groups of subunits within the grey-dotted box:
$Vps27_{yeast}/HRS_{mammals}$ and
$Hse1_{yeast}/STAM1-2_{mammals}$(Stoechiometry 1:1). The grey arrow represents the interaction between Vps27/HRS and the UEV domaine of Vps23/TSG101 that mediates the recruitment of ESCRT-I subcomplex.The orange arrow represents the interaction between Vps27/HRS and PI3P. Based on [Teis 2009].

Last to be characterized, The ESCRT-0 complex is likely to be the first in action during MVB biogenesis. It is composed of two subunits, $Vps27_{yeast}/HRS_{mammals}$[1] and HseI[2] which has two homologs in Metazoans, STAM1 and 2[3]. These two subunits interact in a 1:1 ratio making a two braided GAT domains, each of them consisting in two helices from one subunit and one helix from the other subunit [Prag 2007]. See Figure 2.2 for visual representation of ESCRT-0 subcomplex and interactions: 1- between subunits, 2- between subunits and lipids, 3- between subunits and ubiquitin, 3- with ESCRT-I subcomplex.

Both Vps27/HRS and HseI/STAM1&2 subunits contain a VHS[4] domain of unknown function, an Ubiquitin binding domain and a clathrin binding domain [Hofmann 2001, Raiborg 2001a, McCullough 2006]. HRS contains an additional FYVE[5] zinc finger domain that binds to phosphatidylinositol 3-phosphate (PtdIns3P). It provides both membrane binding and endosomal specificity to the complex. Indeed, PtdIns3P is restricted, to our knowledge, to early endosomes as it is produced *in situ* by the class III PI3 kinase Vps34 [Gaullier 1998, Gillooly 2000, Mao 2000, Raiborg 2001b]. Binding to PtdIns3P containing membranes and to ubiquitinilated cargo confers to ESCRT-0 a great capability to detect and bind to endosomes and select cargo. Besides, both Vps27/HRS and STAM bind directly to Clathrin [Raiborg 2001a, McCullough 2006]. The binding of ESCRT-0 to Clathrin results in flat Clathrin coated membrane domains that seem important for the sorting of cargo at MVBs [Raiborg 2006, Sachse 2002].

ESCRT-0 is not found in plants [Leung 2008]. This may indicate that there are

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1. HRS stands for Hepatocyte growth factor-Regulated tyrosine kinase Substrate
2. HseI stands for Heparanase
3. STAM stands for signal transducing adaptor molecule (SH3 domain and ITAM motif)
4. VHS stands for Vps27, HRS, STAM
5. FYVE stands for Fab1p, YOTP, Vac1p, EEA1

other proteins able to fulfill HRS and STAM roles. Proteins like TOMs[6] or GGAs[7] are good candidates. Indeed, they all contain Ubiquitin binding and Clathrin binding domains [Puertollano 2004, Puertollano 2005], in addition to their possible capacity to bind PtdIns3P binding proteins [Seet 2004].

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6. TOM stands for Target Of Myb
7. GGA stands for Golgi-associated, gamma-Adaptin ear containing, ARF binding protein

1.1.2 The ESCRT-I complex
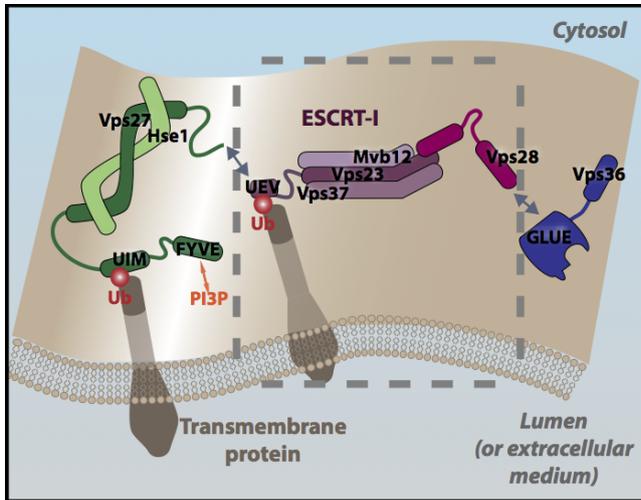
FIGURE 1.3 – **The ESCRT-I complex**
The ESCRT-I subcomplex corresponds to the group of subunits within the grey-dotted box: MVB12$_{yeast}$/MVB12A & MVBB$_{mammals}$, Vps23, Vps37 and Vps28 (Stoechiometry 1:1:1:1). The grey arrows represents the interaction between Vps27/HRS and the UEV domaine of Vps23/TSG101 that mediates the recruitment of ESCRT-I subcomplex, and the interaction between Vps28 and Vps36 mediating the recruitment of ESCRT-II. Based on [Teis 2009].

Identified in yeast prior to the identification of the other ESCRT complexes, ESCRT-I was first characterized as a complex composed of three subunits: Vps23$_{yeast}$/TSG101$_{mammals}$ [8], Vps28 and Vps37 which has four homologs in mammals [Katzmann 2001, Bache 2004, Bishop 2001, Eastman 2005, Stuchell 2004]. Crystal structure showed that these subunits form a complex with a 1:1:1 stoichiometry [Kostelansky 2006, Teo 2006]. See Figure 2.3 for visual comprehension of ESCRT-I subcomplex and interactions: 1- between subunits, 2- between subunits and ubiquitin, 3- with ESCRT-0 and ESCRT-II subcomplexes.

A fourth component MVB12$_{yeast}$/MVB12A and MVBB$_{mammals}$ [9] in Metazoans was later identified as another ESCRT-I complex subunit [Audhya 2007, Morita 2007a] that makes a 1:1:1:1 complex with the three first identified subunits [Kostelansky 2007]. This subunit interacts with the coil-coiled Vps23 and is required for oligomerization and further release of the ESCRT-I complex [Chu 2006, Curtiss 2007].

The interaction of ESCRT-I with membranes being weak, ESCRT-0 is necessary for ESCRT-I recruitment to endosomal membranes [Bache 2003b, Bache 2003a, Katzmann 2003, Lu 2003]. The ESCRT-I complex is recruited by the UEV [10] domain located at the N-terminus of Vps23. Via this domain, Vps23/TSG101 binds both to ubiquitinilated proteins and to the PTAP-like motif (PTVP$_{yeast}$/PSAP$_{mammals}$) of Vps27/HRS (ESCRT-0) [Katzmann 2003, Bache 2003a, Kostelansky 2006]. This interaction is mimicked by the Gag [11] protein in virus *via* a P(S/T)AP motif and used by the virus for budding [Pornillos 2002a, Pornillos 2002b]. In addition to Vps23/TSG101, the discovery of an ubiquitin binding domain inside the protein MVB12 suggests that

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8. TSG101 stands for tumor susceptibility gene 101
9. MVB12 stands for Multi Vesicular Body 12
10. UEV stands for Ubiquitin E2 Variant
11. Gag stands for Group-specific AntiGen

this protein could also participate to the recognition and sorting of ubiquitinilated proteins [Shields 2009].

The C-terminal domain of Vps28 interacts with the GLUE [12] of the ESCRT-II subunit Vps36/EAP45 [13]. This interaction allows the recruitment of the third complex: ESCRT-II [Slagsvold 2005, Teo 2006, Gill 2007].

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12. GLUE stands for GRAM-like ubiquitin-binding in EAP45
13. EAP stands for ELL-Associated Protein

1.1.3 The ESCRT-II complex
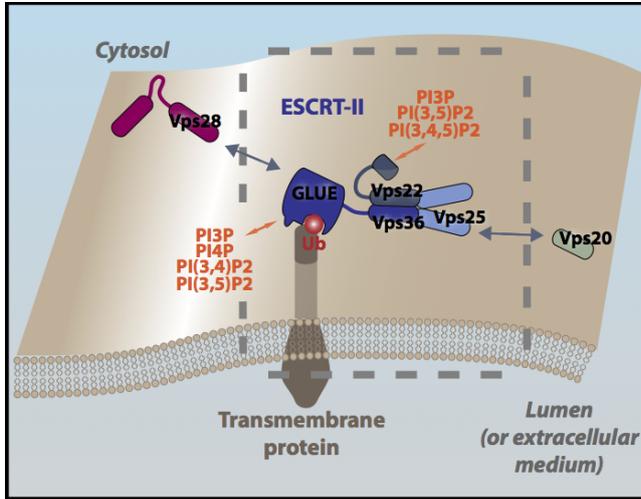
FIGURE 1.4 – **The ESCRT-II complex**
The ESCRT-II subcomplex corresponds to the groups of subunits within the grey-dotted box: Vps22, $Vps36_{yeast}/EAP45_{mammals}$ and $Vps25_{yeast}/EAP20_{mammals}$ (Stoechiometry 1:1:1). The grey arrows represent the interaction between Vps28 and the GLUE domain of Vps36 that mediates the recruitment of ESCRT-II subcomplex, and the interaction between Vps25 and VPS20 mediating the recruitment of ESCRT-III. The orange arrows and letters represents the interaction between Vps36 or Vps22 and the lipids indicated. Based on [Teis 2009].

The ESCRT-II complex is composed of one $Vps36_{yeast}/EAP45_{mammals}$, one Vps22 and two $Vps25_{yeast}/EAP20_{mammals}$ proteins that form a Y-shaped structure [Hierro 2004, Teo 2004]. See Figure 2.4 for visual comprehension of the ESCRT-II subcomplex and interactions: 1-between subunits, 2- between subunits and lipids, 3- between subunits and ubiquitin, 4-with ESCRT-I and ESCRT-III subcomplexes

Vps36/EAP45 represents the key subunit for specific ESCRT-II recruitment. The mammaliam EAP45 binds to the C-terminus of Vps28 (ESCRT-I), ubiquitinated receptors and PtdIns3P (and PtdIns(3,5)P$_2$) via its GLUE domain, in a non competitive manner [Slagsvold 2005, Alam 2006, Hirano 2006, Im 2008].

This domain was identified in yeast by homology with the mammalian protein but it additionally contains two inserted NZF zinc-finger domains, one of which binds Vps28 and a second that binds ubiquitin. Its crystal analysis revealed that it corresponds to a split PH [14] domain with a non canonical lipid binding pocket. It ensures its binding not only to PtdIns3P but also to PtdIns4P, PtdIns(3,4)P$_2$ and PtdIns(3,5)P$_2$ [Kostelansky 2006, Teo 2006, Gill 2007] which are lipids present in late endosomes and in the plasma membrane. See for review on lipid localization [van Meer 2008].

The lipid-binding properties of the first helix of Vps22, are likely to participate with the GLUE domain of Vps36 to the recruitment of the ESCRT-II complex to membranes. The deletion of this helix does not reduce binding of the complex to PtdIns3P, but reduces in the same amount than GLUE deletion the binding to PtdIns(3,5)P$_2$ and PtdIns(3,4,5)P$_3$ [Im 2008].
The binding of Vps25/EAP20 on Vps20/CHMP6(ESCRT-III) ensures the recruitment and the assembly of the ESCRT-III complex [Teo 2004, Teis 2008, Teis 2010].

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14. PH domains are Phosphoinosotide binding domains. PH stands for Pleckstrin Homology

1.1.4 The ESCRT-III complex

The ESCRT-III complex is conserved among opisthokonts [15] [Obita 2007, Hobel 2008, Leung 2008]. In yeast the ESCRT-III complex is composed of four subunits: Vps20, Snf7/Vps32, Vps24 and Vps2 [Babst 2002a]. See Figure 2.5 for visual comprehension of the ESCRT-III subcomplex and interactions: 1-between subunits, 2-between subunits and lipids, 3-with ESCRT-II and ESCRT-disassembly subcomplexes.

The mammalian version of this complex contains more subunits as there are three homologs for Snf7/Vps32 commonly called CHMP4 A, B and C [16], and two homologs for Vps2 commonly called CHMP2A and B.

We can add to the ESCRT-III complex the two "accessory" ESCRT-III-like proteins $Did2_{yeast}$/CHMP1$_{mammals}$ and $Vps60_{yeast}$/CHMP5$_{mammals}$ that are likely either to be redundant (as their inactivation results in a milder phenotype than the deletion of the "core" subunits of the ESCRT-III) or to play a regulatory role, especially during the recruitment of Vps4

FIGURE 1.5 – **The ESCRT-III complex**

A- The ESCRT-III subcomplex corresponds to the groups of subunits within the grey-dotted box: $Vps20_{yeast}$/CHMP6$_{mammals}$, Snf7(or $Vps32)_{yeast}$/CHMP4A, B and C$_{mammals}$, $Vps24_{yeast}$/CHMP3$_{mammals}$ and $Vps2_{yeast}$/CHMP2A and B$_{mammals}$ (Stoechiometry not known). The grey arrows represents the interaction between Vps25 and vps20 that mediates the recruitment of ESCRT-III subcomplex, and the interaction between Vps2 and VPS4-Vta1 disassembly complex. The orange arrows and letters represents the interaction between Snf7 or Vps24 and the lipids indicated. B- Release of the autoinhibition of ESCRT-III subunits and polymerization. Based on [Teis 2009].

[Amerik 2000, Babst 2002a, Bajorek 2009a].

Similarities in crystal structures of CHMP3 and an "accessory" ESCRT-III protein IST1 [17] [18] suggest that ESCRT-III subunits share a close architecture [Muziol 2006, Bajorek 2009a, Bajorek 2009b].

There is evidence that the oligomerization of the subunits is regulated by autoinhibitory sequences located in the vicinity of their acidic C-terminus [Lin 2005,

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15. Opistokhonts is a broad group of eukaryots including Fungi and Metazoans
16. CHMP stands for charged multuvesicular body proteins
17. IST stands for increased sodium tolerance
18. IST-1 is required together with Did2/CHMP1 for efficient Vps4 recruitment and abscission during cytokinesis in HeLa cells, but is not requird for HIV budding [Bajorek 2009a]

Muziol 2006, Zamborlini 2006, Shim 2007, Lata 2008a]. The subunits remain in a metastable [19] soluble conformation until binding of a partner protein. This will release the autoinhibition and favor oligomerization.

Therefore, the binding of Vps25 (ESCRT-II) on the C-terminus of Vps20 is the event that marks the recruitment of ESCRT-III by ESCRT-II complex, and the begining of the oligomerization of ESCRT-III subunits [Teo 2004]. From here on, the last recruited subunit will bind in a competitive manner to the next's subunit C-Terminus, activating the last for the recruitment of another subunit and for the binding to membranes through its now free basic N-terminus [20] [Lin 2005, Muziol 2006, Zamborlini 2006, Shim 2007].

Besides, ESCRT-III proteins can also bind to membranes thanks to additional phosphoinositide binding domains that have been identified in most subunits. Vps20/CHMP6 binds to membranes through a myristyl group in its N-terminal region [Yorikawa 2005]. The N-terminal domain in Snf7 binds to phosphoinositides and specially to PtdIns3P and PtdIns$(3,5)$P$_2$ [Lin 2005] but it is not clear yet how Snf7 binds to lipids precisely. Vps24/CHMP3 binds to lipids through a basic motif in the N-terminal domain mostly to PtdIns$(3,4)$P$_2$ and PtdIns$(3,5)$P$_2$. This motif is conserved among all ESCRT-III proteins including CHMP4A/hSnf7-1 and could thus be the motif used by ESCRT-III proteins to bind to membranes [Whitley 2003]. The work of S. Saksena and co-workers suggests that the assembly of ESCRT-III subunits requires prior binding to membranes [Saksena 2009b].

The stoichiometry of the complex is not known but it is likely that the proportion of subunits forming the complex is variable. Snf7 is about 10 times more abundant than the other subunits in the complex [Teis 2008]. Studies *in vivo* and *in vitro* have shown that the subunits of ESCRT-III also assemble on MVBs following a specific order [Teis 2008, Wollert 2009]. First of all, Vps20 is recruited via its interaction with the ESCRT-II subunit Vps25 as described above. Vps20 then nucleates the oligomerization of Snf7 [Lin 2005]. It is possible that more than one Snf7 filament is generated through the nucleation of Snf7 by the two armed ESCRT-II complex (Y-shaped) as proposed by [Teis 2010]. Additionally, further data from D. Teis and co-workers suggest that oligomerization of Snf7 would espacially trap cargo at the endosomal membranes [Teis 2008]. The oligomerization of Snf7 is terminated by the recruitment of the subcomplex Vps24-Vps2 via the interaction of Vps24 with Snf7. This subcomplex then participates in the recruitment of Vps4 to initiate ESCRT-III disassembly [Teis 2008, Teis 2010].

The *in vitro* study from T. Wollert, C. Wunder and co-workers provided evidence that the subunits Vps20, Snf7 and Vps24 added on the extra-GUV medium were sufficient to release vesicles into the lumen of the GUVs. Vps2 and Vps4 were necessary for recycling of the subunits and therefore for subsequent rounds of inner vesicles formation [Wollert 2009].

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19. As proposed by [Lata 2008a] since its soluble conformation can be changed by ionic strength, which is consistent with an electrostatic interaction between an acidic C-terminus and a basic N-terminus as proposed by [Whitley 2003]

20. The removal of the C-terminal region results in spontaneous membrane targeting [Lin 2005, Muziol 2006]

1.1.5 The ESCRT-disassembly complex
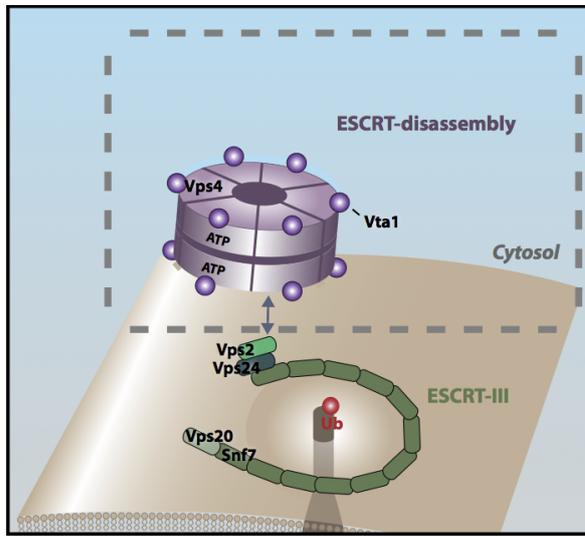
FIGURE 1.6 – **ESCRT-disassembly complex**

The ESCRT-disassembly complex corresponds to barrel within the grey-dotted box. It is composed by Vps4 and Vta1. The grey arrows represents the interaction between Vps2 and Vps4/Vta1. Based on [Teis 2009].

The disassembly of ESCRT-III complexes is ensured by Vps4, which belongs to the class1 AAA+ [21] ATPase family [Babst 1997, Babst 1998]. This enzyme is conserved both in eukaryotes and archea, testimony of its importance [Leung 2008, Obita 2007, Hobel 2008, Lindas 2008, Samson 2008]. Vps4 contains a central AAA domain responsible for its ATPase catalytic activity, a N-terminal MIT [22] domain that binds ESCRT-III subunits, and a novel C-terminal domain which is also required for the recruitment of Vps4 to endosomal membranes and binding to Vta1/LIP5 [Babst 1998, Yeo 2003, Scott 2005a]. See Figure 2.6 for visual comprehension of the ESCRT-disassembly subcomplex and interactions: 1-between subunits, 2- with ESCRT-III subcomplex.

Vps4 is recruited through its interaction with ESCRT-III subunits via the MIT domain of Vps4 and MIM (MIT Interacting motif) motif of ESCRT-III proteins. MIM1 motifs are found at the extreme C-terminus of Vps2 and Vps24, and MIM2 motifs are found in the C-terminal regions of Vps20 and Snf7 [Yeo 2003, Fujita 2004, Lin 2005, Obita 2007, Stuchell-Brereton 2007, Shim 2007, Kieffer 2008, Shim 2008]. MIM1 and MIM2 motifs interact in different subdomains of the MIT domain allowing simultaneous interactions. Both interactions have been shown to be important for Vps4 activity but it is not clear whether MIMs domains from all ESCRT-III proteins are functional. It is likely that the recruitment of Vps4 to ESCRT-III subunits promotes Vps4 oligomerization, as cytoplasmic oligomers of Vps4 have not been detected [Babst 2002a, Fujita 2004].

Vps4 forms multimers of 12 subunits that assemble into a double layer of hexameric rings. Its multimerization is ATP-dependent. Additionally, the active complex contains the protein Vta1/Lip5 which binds with more affinity once the Vps4 multimer is formed, via the C-terminus of each Vps4 and Vta1 [Yeo 2003, Fujita 2004, Scott 2005a] in a ratio 2(Vps4) : 1(Vta1) [Scott 2005a, Yu 2008], probably only in the presence of ATP [23] [Azmi 2006]. Vta1's interaction with several ESCRT-III subunits (CHMP1, CHMP2A, CHMP3, CHMP5 but not CHMP4A and CHMP6) indicates that this protein may

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21. AAA stands for ATPase associated with varius cellular activities
22. MIT stands for Microtubule Intercating and Transport
23. The interation of the ATP hydrolysis mutant Vps4-(E233Q) with Vta1 was decreased in experiments performed by [Azmi 2006] and enhanced in experiments from [Yeo 2003]

have a role in specificity of Vps4 recruitment. Vta1 interacts, as Vps4, with the MIM domains of CHMP1 and 2A [Bowers 2004, Ward 2005, Shim 2008]. Nevertheless, an additional Vps4 binding site [24] was discovered in these proteins within the alpha5 helix, suggesting a possible simultaneous binding of Vps4 to ESCRT-III, and Vps4 to Vta1[Shim 2008].

Additionally, the IST1 and Did2/CHMP1B complex, is important for the recruitment of Vps4 on the ESCRT-III complex [Nickerson 2006, Rue 2008]. Did2 is recruited to endosomes via its interaction with the Vps2-Vps24 subcomplex [Nickerson 2006] and recruits, via its MIM binding domain, Vps4 (binding to Vps4 MIT domain) [Yeo 2003, Shiflett 2004, Scott 2005b, Lottridge 2006, Azmi 2006, Nickerson 2006]. It controls the size of the intraluminal vesicles in MVBs probably by timing Vps4 recruitment [Nickerson 2010]. Did2 interacts with Vta1 and is necessary for Vta1 interaction with snf7/CHMP4 [Lottridge 2006, Azmi 2008]. IST1 is recruited to the ESCRT complex via its partner Did2 [Rue 2008, Dimaano 2008, Xiao 2009]. It interacts with Vps4 so that it cannot bind to the ESCRT machinery controlling in that way ESCRT-III disassembly [Dimaano 2008]. Vps 4 actually recruits Vta1 once the subcomplex Did2-IST1-Vps4 is formed [Shestakova 2010].

$Vps60_{yeast}$/$CHMP5_{mammals}$ is probably involved, like Vta1, in later stages of Vps4 activation, and it has been shown to promote efficient membrane scission [Nickerson 2010]. The recruitment of Vps60 is dependent on its interaction with Vta1 but also on Vps4 [Shiflett 2004, Rue 2008, Azmi 2008]. Neither Vps60 nor Vta1 depletion have strong impact on Vps4 activity. The phenotype of Vps4 depletion is only phenocopied when combining the depletion of Did2 and Vta1 or Did2 and Vps60 [Azmi 2006, Nickerson 2010]. Nevertheless, in the absence of Vta1, Vps4 exhibit a minimal ATPase activity. The presence of Vta1 actually increases Vps4 enzymatic activity by 6 to 8 fold [Yeo 2003, Lottridge 2006, Azmi 2006] probably by promoting and stabilizing the correct folding of the Vps4 oligomer already assembled with IST1 and Did2 [Azmi 2006].

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24. It is not clear if Vps4 binds to this secondary site directly or via an adaptor protein

1.2 The ESCRTs: role in cellular functions and versatility of the machinery's actors
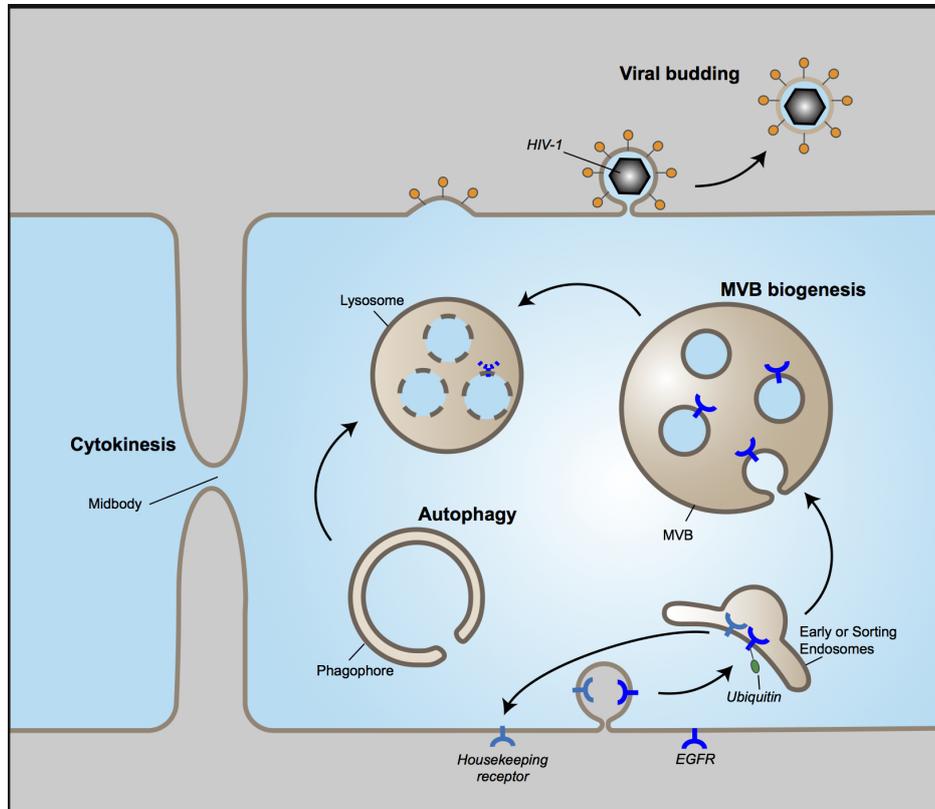
FIGURE 1.7 – **The role of ESCRTs in cellular processes.**
ESCRTs are implicated in several processes in the cell, mediating the formation of buds with similar topologies. They are involved in: 1- ILV formation, 2- Virus budding, 3- Cytokinesis, 4- Autophagosome closure (hypothetical). Based on [Hurley 2010]

First identified as MVB biogenesis proteins, ESCRTs were later implicated in other three defined cellular functions: cytokinesis, viral budding and autophagy. These functions brought interesting understanding about ESCRT complexes. In this section, I will present details about the involvement of ESCRTs in the different processes enumerated above. I will also discuss the differences in ESCRT actors and some associated proteins for each of these processes. Furthermore, the mechanisms by which ESCRTs create membrane deformation and scission will be discussed.

1.2.1 The ESCRTs in virus budding
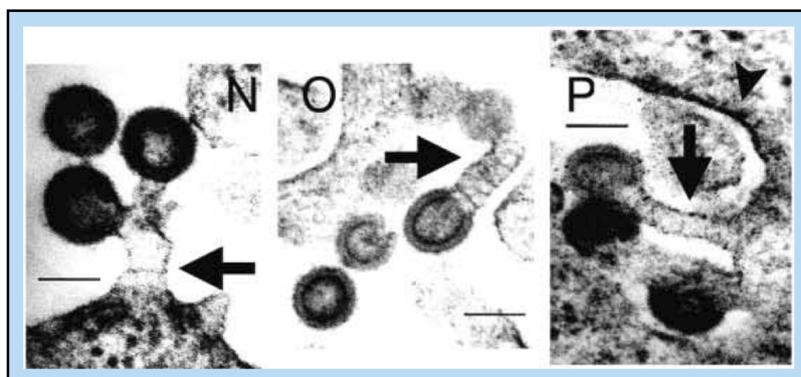
FIGURE 1.8 – **Ultrastructure of HIV-1 budding**
These pictures correspond to electron micrographs of HEK293T cells transfected with HIV-1 particle forming constructs. The black arrows show striations in the necks of the buds. Those striations could correspond to ESCRT helixes. Adapted from [von Schwedler 2003].

Shortly after the discovery of ESCRTs, J. E. Garrus and co-workers discovered a novel function for these proteins in virus budding thanks to the following observation: a decreased expression of TSG101 or expression of the dominant negative mutant of Vps4 inhibited HIV-1 and MLV [25] budding [Garrus 2001, Demirov 2002]. It was also shown for HIV-1, MLV and Ebola Virus that TSG101 could bind through its UEV domain [26] to the PTAP tetrapeptide motif in the viral Gag [27] protein in a direct fashion and that this interaction seems essential for virus budding [Garrus 2001, VerPlank 2001, Martin-Serrano 2001, Pornillos 2002a].

The PTAP motif belongs to what is called the L-domains (late-domains) which are short motifs including P(S/T)AP, YP(X)nL and PPXY encoded by Gag proteins which have been described as essential motifs for virus release [Craven 1999, Harty 1999, Harty 2000]. They are found in several viral genomes such as the ones of retrovirus [28], filovirus, rhabdovirus, arenavirus and paramyxovirus [29] families [Bieniasz 2006]. These domains were shown to be important for budding of arenavirus and paramyxovirus and this last in a TSG101 dependent fashion [Perez 2003, Schmitt 2005].

Besides, some L-domains recruit ESCRT machineries through different means, and not only through interaction with TSG101. LYPXnL L-domains binds to the ESCRT-III associated protein ALIX, which in turn recruits ESCRT through its interaction with CHMP4 proteins [von Schwedler 2003, Fisher 2007, McCullough 2008, Strack 2003, Usami 2007, Zhai 2008]. Furthermore, HIV-1 contains, in addition to its binding site for TSG101, two binding sites for ALIX encoded within the Gag protein [Strack 2003, Popov 2008]. ALIX can also interact with TSG101 and with CHMP6 [von Schwedler 2003], nevertheless the domain of interaction with TSG101 seems not

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25. HIV stands for Human Immunodeficiency Virus; MLV stands for Murine Leukemia Virus
26. As a reminder, TSG101 interacts with ubiquitinilated cargo and with HRS via UEV domain
27. Gag stands for group-specific antigen
28. Retrovirus belong to Groupe VI, which have a simple brand positive RNA
29. Filovirus (to which Ebola virus belongs), rhabdovirus, arenavirus and paramyxovirus are viruses belonging to Groupe V, with a genome constituted of simple strand negative RNA

to be crucial for HIV-1 budding [Usami 2007] The capacity of interaction with several ESCRT proteins likely ensures a more efficient recruitment of the machinery. Besides, ESCRT-III recruitment through ALIX, bypassing ESCRT-0, ESCRT-I and ESCRT-II, indicates that these sub-complexes may be dispensable for some ESCRT functions. The expression of dominant-negative mutants of CHMP3, CHMP4B and CHMP4C or the catalytically inactive mutant of VPS4 inhibits hepatitis [30] and herpes simplex virus [31] release [Crump 2007, Lambert 2007, Watanabe 2007]. Also, deletion of autoinhibitory domains result in HIV budding increase revealing the importance of ESCRT-III sub-complex in virus release [Zamborlini 2006].

Nevertheless, Lambert and co-workers showed that expression of the dominant negative mutants mentioned above triggered trapping of viral envelope of hepatitis B virus in endosomal vacuoles [Lambert 2007]. Thus, in the case of hepatitis B virus, it is not clear whether the impairment of ESCRTs avoids budding at the plasma membrane or rather inhibits upstream steps like virus particle assembly. It may also suggest that virus budding, or at least the first steps of the process, could occur at the endosomes that would subsequently fuse with the plasma membrane to release the virus [Lambert 2007]. Another hypothesis is that endosomal compartments where the viruses are found could already be connected to the extracellular medium through microchannels as it is proposed in the case of HIV-1 infection of macrophages. See [Benaroch 2010] for review. In this case, they would be no need to bud at the plasma membrane but only in the endosomes and specially MVBs. Therefore, inhibiting the MVB biogenesis would inhibit the whole process of virus production and release. Also, T. Watanabe and co-workers showed that ALIX dominant negative mutant inhibited budding but not capside assembly of hepatitis B virus, whereas Vps4B dominant negative mutant inhibited both steps [Watanabe 2007]. However, Vps4 has been shown to be involved in the recycling of ESCRT subunits but not in budding itself [Wollert 2009]. The absence of budding of hepatitis B virus when Vps4B dominant negative is expressed may be explained by an inhibition of the whole ESCRT-III because of the absence of recycling of the subunits, which become unavailable. Another explanation is that virus with defective assembly of their capside cannot undergo budding.

The hijacking of ESCRT machineries by viruses brings us valuable information on ESCRT mechanisms and especially concerning the versatility of the system for differential implication of ESCRT subunits. See [McDonald 2009] for review.

1.2.2 The ESCRTs in cytokinesis

During the late stages of mitosis, after the distribution of genetic material and organelles has been completed, the two daughter cells remain attached to each other through a cytoplasmic bridge. The severing of this bridge is called abscission, and marks the very end of the mitotic process. This last step corresponds to a coordinated work of several proteins, signals and processes including vesicle targeting, vesicle fusion and membrane constriction and scission. Some of the actors of this process are presented in

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30. Hepatitis virus belongs to the picornavirales which belongs to the Group IV, characterised by a single-positive RNA genome

31. Herpes virus belongs to the Group I, characterised by DNA genome
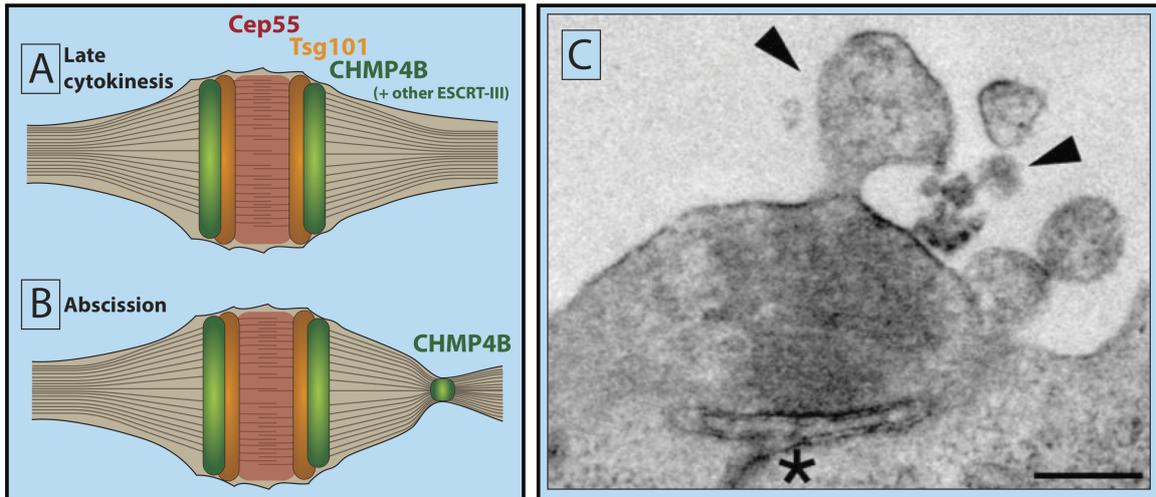
FIGURE 1.9 – **Spatial organization of ESCRT proteins at the cytokinetic bridge**

A- and B represent the localization of some ESCRT proteins in the midbody in late cytokinesis and during abscission [Elia 2011]. C-Membrane-buds observed in the midbody membrane of neuroendocrine cells. Adapted from [Dubreuil 2007]

Table 2.1, see [Fededa 2012] for review. I would like to point out the crucial role for Exocyst and SNAREs, that shows the importance of membrane fusion in this process, probably between vesicles, and between vesicles and the plasma membrane. In previous sections I presented the importance of exocytosis as a membrane supplier and a tension modulator. It is very likely that exocytic events in the cytoplasmic bridge have a crucial role to decrease tension and to facilitate membrane deformation and scission.Yet, we cannot exclude that SNAREs may play a role in membrane fusion. About the trafficking toward the cytoplasmic bridge see review [Prekeris 2008]. Nevertheless, the topology of the membranes in the cytoplasmic bridge resembles the topology of the membranes in the neck of virus budding vesicles and ILVs. Recent work begins to test the model whereby ESCRTs would mediate the fusion of membranes during the severing of the cytokinetic cytoplasmic bridge.

ALIX (ESCRT-III related protein) and TSG101(ESCRT-I protein) localize in the central region of the midbody [Carlton 2007, Morita 2007b], and CHMP2A, CHMP4A, CHMP4B, CHMP5 and Vps4 (ESCRT disassembly) are located on both sides of the midbody [Morita 2007b]. See Figure 2.9 for visual comprehension of the localization of some ESCRT subunits at the midbody. Recent work using improved imaging resolution could show that TSG101 is located more towards the center of the midbody than CHMP4B but is nevertheless on both sides of the more central structure of the midbody called the Flemming body [Elia 2011]. CHMP3 is also recruited at the midbody but its precise localization relative to the other recruited ESCRT has not been studied [Dukes 2008]. The interaction of TSG101 with Vps28 (ESCRT-I) is also crucial for abscission indicating that probably the whole ESCRT-I complex is implicated in abscission [Carlton 2007]. The order of recruitment of the different ESCRT proteins involved in late steps of cytokinesis at the cytoplasmic bridge is

Protein/Complex	Role	References (to complete)
Centriolin and γ-tubulin	Form the "midbody ring", which recruits the vesicle targeting proteins of the Exocyst.	(Gromley et al. 2003; Gromley et al. 2005)
The v-SNARE endobrevin/VAMP8 and the t-SNARE syntaxin-2	Recruited by components of the midbody ring for facilitating membrane fusion, probably between vesicles and between vesicles and the plasma membrane. This phenomenon is crucial for abscission.	(Gromley et al. 2005; Low et al. 2003)
The endocytic Rab GTPase proteins: Rab35, Rab8 and Rab11	Involved in the regulation of vesicle trafficking during cytokinesis, in coordination with other proteins such as some Rab11 interacting proteins FIP3 and FIP4, and Arf6	Fielding et al. 2005; Kouranti et al. 2006; Pohl et al. 2008; Wilson et al. 2005; Yu et al. 2007; Prekeris et al. 2008)

Table 1.1 – **Some actors of cytokinesis**

References: [Gromley 2003, Low 2003, Gromley 2005, Fielding 2005, Kouranti 2006, Pohl 2008, Wilson 2005, Yu 2007, Prekeris 2008].

compatible with *in vivo* studies and *in vitro* reconstitution of the ESCRTs recruitment [Teis 2008, Wollert 2009, Wollert 2010].

Depletion of ESCRT-III by siRNA or expression of dominant-negative construct inhibits abscission [Dukes 2008, Morita 2007b] and ALIX binding site in ESCRT-III is crucial for cytokinesis [Carlton 2008]. Importantly, the over-expression (which at hight levels causes a dominant-negative effect) of CHMP4A, CHMP4B and CHMP4C has different effects on HIV-1 release and cytokinesis. The over-expression of CHMP4B has a stronger inhibitory effect for HIV budding and the over-expression of CHMP4C has a stronger inhibitory effect for cytokinesis compared to the over-expression of CHMP4B [Carlton 2008, Carlton 2012]. Suprinsingly, at low levels CHMP4C also delays abscission through phosphorylation of AuroraB [Carlton 2012] [32]. Additionally, siRNA against CHMP4C did not inhibit absicission, suggesting that the over-expression of CHMP4C may inhibit abscission by enhancing its role of abscission regulator or by inducing a dominant negative effect on other ESCRT subunits especially on its paralogue CHMP4B which is required for abscission [Dukes 2008, Morita 2007b, Carlton 2012]. The fact that CHMP4B and CHMP4C have opposite effects on absission suggests that different isoforms of the yeast homologues CHMP proteins may not have redundant roles in mammals and may be involved in different ESCRT-mediated processes.

Furthermore, TSG101 and ALIX bind to the midbody protein CEP55, which is located at the Flemming body [Fabbro 2005, Zhao 2006, Elia 2011] and is crucial for abscission [Fabbro 2005, Martinez-Garay 2006, Zhao 2006]. TSG101 and ALIX compete for both ESCRT and CEP55 binding. A model was proposed in which several dimers CEP55-ALIX and CEP55-TSG101 are required for ESCRT recruitment [Martinez-Garay 2006, Lee 2008]. ALIX binds to CEP55 through the PRR region in ALIX, nevertheless other modes of recruitment of ESCRT machineries must exist

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32. AuroraB is a serine-threonine-protein kinase involved in the stabilization of the central spindle and overlapping microtubules at the midbody during mitosis.

in other organisms as this binding site is not found in *Saccharomyces cerevisae*, in *Caernorhabditis elegans* or in *Drosophila melanogaster* [Morita 2007b, Carlton 2008]. Moreover, interaction between TSG101 and ALIX, and CEP55 is crucial for abscission and not for virus budding [Carlton 2007, Morita 2007b, Carlton 2008].

In a similar fashion, IST-1 is required together with Did2/CHMP1B [33] for efficient Vps4 and Vta1 recruitment and abscission during cytokinesis in HeLa cells, but is not required for HIV budding [Agromayor 2009, Bajorek 2009a]. hIST1 is also localized to both sides of the Flemming body [Agromayor 2009, Bajorek 2009a]. Both IST1 and CHMP1B also interacts with spastin [Reid 2005, Agromayor 2009], which is also an ATPase (like Vps4) and a microtubule severing enzyme, and this interaction is necessary for cytokinesis [Connell 2009, Yang 2008] . It is possible that IST-1 and CHMP1B coordinate the severing of microtubules by spastin. They are likely to obstruct membrane proximity and the membrane fusion mediated by ESCRTs [Connell 2009]. Importantly, work in Crenarchea demonstrated that homologues of ESCRT-III and Vps4 among other ESCRT proteins exist in this family [Obita 2007, Hobel 2008]. The expression of a catalytically inactive Vps4 mutant triggered failure of cell-division [Lindas 2008, Samson 2008] suggesting an evolutionary conserved mechanism.

However, the hypothesis that ESCRTs mediate cytoplasmic bridge contriction in a similar fashion than they operate constriction during virus budding or ILV biogenesis still generate doubts. Indeed, the diameter of the neck during ILV or virus budding measures 50-100nm in diameter whereas the cytoplasmic bridge is about 1.5 - 2.0 μm in diameter [Gromley 2005]. Several models try to explain the *modus operandi* of ESCRTs in cytokinesis. The first one is based on the observation that CHMP4B recruitment occurs at both sides of the Flemming body but also later on one side of the cytoplasmic bridge. See Figure 2.9 - panel B. The diameter of the bridge at this spot is smaller and therefore more compatible with the diameters of other necks where the ESCRT complex plays a role in constriction [Guizetti 2011, Elia 2011]. It is also likely that ESCRTs mediates the narrowing of the diameter of the cytoplasmic bridge, adapting to bigger diameters than the ones that have been described before, as J. Guizetti and co-workers showed the presence of ESCRT-III-dependent helix-like filaments in the same zone where ESCRT-III proteins were recruited [Guizetti 2011]. The narrowing of the bridge could also be ensured by the removal of membrane by budding [Dubreuil 2007]. See Figure 2.9 Pannel C. Other models include the fusion of vesicles with the plasma membrane mediated by SNAREs [McDonald 2009] leading to a "wasp waist" shape in the cytoplasmic bridge, or the processing of several necks between juxtaposed vesicles mediated by ESCRTs or by both ESCRTs and SNAREs. The cytoskeleton could also play an important role in narrowing the cytoplasmic bridge to make it more compatible with ESCRT activity.

1.2.3   The ESCRTs in autophagy

Recent work has proposed a novel role for ESCRT in macroautophagy. See [Rusten 2009] for review. Macroautophagy is a degradative process were cytosolic com-

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33. As a reminder Ist-1 and CHMP1 are ESCRT-III associated proteins
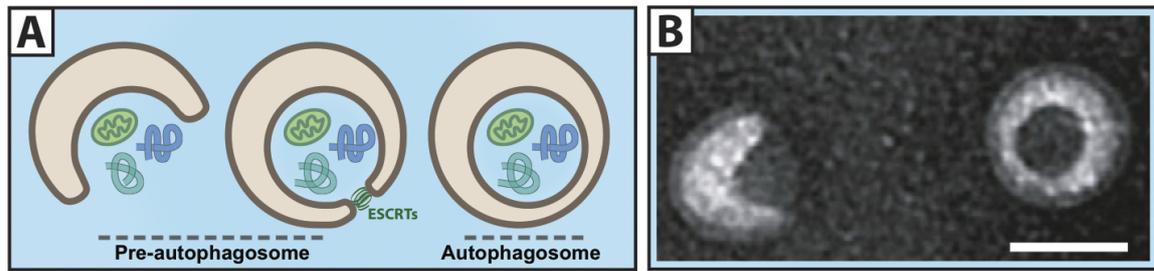
FIGURE 1.10 – **Model for autophagosome closure**

A- Model for autophagosome closure mediated by ESCRTs [Rusten 2009]. B- Invaginations seen in PS/PC/PI liposomes incubated with ESCRT proteins and analyzed by negative staining EM. Note that the structures observed are very similar to the model. Adapted from [Saksena 2009b].

pounds are engulfed by a double membrane (looking like a collapsed vesicle) growing and closing into itself to form a sealed double membrane vesicle. See [Mizushima 2008] for review. The details of the autophagic process will be given in chapter 4 as autophagic machinery constitute a main subject in this work.

The involvement of ESCRT in autophagosome closure was expected as the neck formed by the pre-autophagosome [34] is topologically similar to the one formed during budding of ILVs, but in this case with a unique invagination. Liposomes with a single inner vesicle have been observed in PS/PC/PI liposomes incubated with ESCRTs [Saksena 2009b]. See Figure 2.10.

Several lines of evidence argue in favor of a role of ESCRT proteins in the regulation of autophagocytosis. First, depletion of ESCRT subunits inhibits the fusion of autophagosomes with endosomes and/or lysosomes [Filimonenko 2007, Rusten 2007]. This consistently leads to the accumulation of non-degradative autophagosomes as shown in Drosophila with the depletion of Vps28 (ESCRT-I), Vps25 (ESCRT-II), Vps32 (ESCRT-III) or Vps4 (ESCRT-disassembly) [Rusten 2007]. Depletion of Vps4 or TSG101 in mammalian cells leads to accumulation of non-mature autophagosomes and proteins aggregates [Filimonenko 2007, Rusten 2007]. HRS (ESCRT-0) depletion in HeLa cells also led to accumulation of non-mature autophagosomes [Tamai 2007].

Second, CHMP4B mutation in rat neurons in culture led to cortical neurons loss, and the expression in Drosophila of CHMP2B[intron5] which sequesters CHMP4B (because of its higher affinity than CHMP2B[wt]), caused the same phenotype [Lee 2007a]. Neurodegeneration caused by CHMP2B[intron5] was delayed and reduced by lowering the expression of Atg5 and Atg7, which are essential proteins for autophagosome generation [Lee 2009] [35]. This suggests that the neurodegeneration phenotype may be caused by the accumulation of autophagosomes. similarly, HRS knock-out mice accumulated proteins such as p62 [36] and presented serious loss of hippocampal CA3 pyramidal

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34. The pre-autophagosome corresponds to the precursor of the autophagosomes which is not fully sealed

35. Another study using the same CHMP2B mutation in hippocampal neurons couldn't observe any changes in autophagy [Belly 2010]. The reason of this difference remains unundersthood

36. p62 is degraded by autophagy and is involved in the elimination of protein aggregates. Its role in autophagy will be described later in chapter 4

neurons [Tamai 2008]. Interestingly, a single mutation in a splicing site in CHMP2B has been associated to a chromosome-3 linked autosomal dominant frontotemporal dementia in a Danish family [Skibinski 2005, Momeni 2006].

It is mostly clear that ESCRT machinery impairment has an impact on autophagosome maturation. Yet, the role of ESCRT in autophagosomal closure remains a hypothesis. Another possibility would be that this defect in autophagosome maturation would be due to the impairment in lysosome biogenesis. This is supported by the fact that the number of lysosomes is strongly reduced in cells with impaired ESCRT machineries [Doyotte 2005, Razi 2006, Filimonenko 2007, Rusten 2007]. The mechanisms responsible for the formation of autophagosomes is still unclear and the formation by elongation and closure of the pre-autophagosomal membrane remains only a model among others. See [Longatti 2009] for review.

1.2.4   Different functions, different subunits required

Recent progress in the understanding of the involvement of ESCRTs in the differents cellular processes has shown that not all the ESCRT subunits and associated proteins are involved in each process.

ESCRT-II is required for ILV formation in yeast but it is debated whether this sub-complex and its binding partner in ESCRT-III (CHMP6/Vps20) are strictly required in mammals [Bowers 2006, Langelier 2006, Malerod 2007]. ESCRT-II depletion by siRNA impairs the degradation of the chemokine receptor CXCR4 and the downregulation of the EGFR [Langelier 2006, Malerod 2007]. Nevertheless, this depletion does not seem to affect the degradation of the major histocompatibility class-I complex (MHC-I) [Bowers 2006]. It is therefore possible that ESCRT-II acts only in the processing of a subpopulation of cargo, and that, in other processes, it is replaced by proteins capable to make the link between ESCRT-I and ESCRT-III sub-complexes. Furthermore, ESCRT-II seems to be dispensable for virus budding [von Schwedler 2003, Langelier 2006]. See [McDonald 2009] for review.

The ESCRT-III associated protein ALIX is required for the budding of certain viruses and for cytokinesis [Carlton 2008] but not for EGFR sorting at the MVBs [Doyotte 2008]. Instead, the ALIX-related protein PTPN23 [37] is required for sorting and for correct ILV biogenesis at MVBs [Doyotte 2008]. Moreover, the interaction between TSG101 and ALIX, and CEP55 is crucial for abscission and not for virus budding [Carlton 2007, Morita 2007b, Carlton 2008]. ALIX multimerization is required for HIV budding but it is dispensable for cytokinesis [Carlton 2008, Pires 2009]

Some other ESCRT-associated proteins present differences. IST-1, for example, is required together with Did2/CHMP1B for efficient Vps4 recruitment and abscission during cytokinesis in HeLa cells, but is not required for HIV budding [Agromayor 2009, Bajorek 2009a]

Moreover, some subunits of ESCRTs are not found in all Metazoan. As discussed previously, ESCRT-0 is not found in plants [Leung 2008]. The role of this sub-complex may be fulfilled by other proteins with similar binding characterisitics such

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37. PTPN23 stands for protein tyrosine phosphatase, non-receptor type 23

as TOM or GGA. These proteins contain Ubiquitin and Clathrin binding domains [Puertollano 2004, Puertollano 2005], and may be able to interact indirectly with PtdIns3P via adaptor proteins [Seet 2004].

Finally, the existance of several homologues of the yeast proteins in humans suggests that the components of the complex may determine the specificity of the complex for particular functions and not for others. Also, within a specific cellular function, the different related proteins may bring specific activities as suggested by pioneer work on CHMP4 proteins, revealing most particularly a role for CHMP4C in delaying abscission during cytokinesis [Carlton 2008, Carlton 2012].

1.3 Cargo recongnition and subsequent membrane deformation and fission driven by ESCRTS

The ESCRT machinery require a three step process [38]: 1- The specific targeting of the ESCRT machinery, 2- The deformation of the target membrane to form a nascent vesicle; 3- The scission of the vesicle. Nevertheless, it is not yet well understood how ESCRT drive membrane deformation, vesicle formation and membrane scission. In this part we will review the arguments and models describing how all these ESCRT proteins are targeted and how they interplay to create this very special topology of budding which is until now characteristic of ESCRT mediated processes.

1.3.1 Cargo recognition and ESCRT targeting

In addition to the capacity to deform membranes, another crucial quality of ESCRT is its capacity to recognize the cargo that needs to be captured in the bud. As we saw in previous sections, several ESCRT subunits have phospholipid binding domains (see Table 1.1), with special affinity to phospholipids that are roughly characteristic of the compartments where ESCRT act (plasma membrane, and early and late endosomes). These potential bindings may confer a compartment specificity to ESCRTs. Furthermore, most functions of ESCRTs ensure the invagination of membranes to create vesicles containing specific cargo (some receptors in the case of MVBs, viral particles in the case of virus budding). Ubiquitination of the cargo and ubiquitin-binding domains in ESCRT subunits are likely to play a key role in cargo segregation. See [Shields 2011] for review.

Ubiquitination - Generalities

Ubiquitination corresponds to the linkage of one or several ubiquitin proteins on a target protein. Depending on their branching and the type of chain they form, the target protein will head to degradation, internalization or will initiate signaling cascades. Ubiquitin is attached to the epsilon-amino group of the target protein via its C-terminal Glycine. Ubiquitin is first charged on a E1-ubiquitin conjugating enzyme in an ATP dependent manner. Ubiquitin is then transferred into an E2-ubiquitin conjugating enzyme which is bound to an E3-ubiquitin conjugating enzyme capable of recognizing the substrate protein. Once E3 binds to the target protein, E2 can transfer the ubiquitin to the target protein. The mechanism of this transfer is not yet well understood. An additional factor, E4, is needed for the formation of polyubiquitin chains [Koegl 1999]. The human genome encodes two E1 enzymes, two E4, 37 E2 enzymes and more than 600 E3 enzymes. It is therefore very likely that E2 and E3 enzymes are the ones making cargo and function selection for ubiquitination.

Concerning polyubiquitin chains, we can distinguish several chain topologies associated to different linking between ubiquitins. Ubiquitin is usually attached to lysine

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38. Although at first view autophagy does not involve budding, the closure of the autophagosome can indeed be assimilated to MVB formation as reviewed by [Fader 2009]
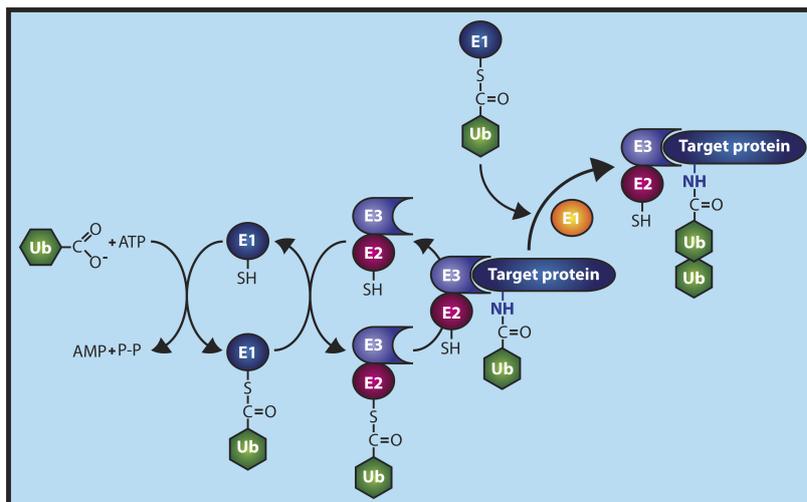
FIGURE 1.11 – **The general mechanism of ubiquitination.**
Ubiquitin binds to E1 in an ATP-dependent reaction. Ubiquitin is transferred to the complex E2-E3.
The E3 protein recognizes to target protein and binds to it. Ubiquitin is transferred to the target
protein. Similar reactions with Ubiquitin as target proteins are possible in the presence of E4. They
lead to a polyubiquitin chain.

residues of the substrate. In a polyubiquitin chain, the substrate is ubiquitin itself. The
different chains are named in reference to the position, in the target Ubiquitin, of the
Lysine where the next Ubiquitin is attached. This rule is increasingly debated as it
is very likely that different kinds of linking may occur inside the same chain. These
heterotypical chains are likely to have different functions.

The general mechanism for ubiquitination is presented in Figure 2.11. The ubiquiti-
nation of a protein can be reverted by du-ubiquitinating enzymes commonly known as
DUBs, which can cleave part or all the polyubiquitin chains.

Role of ubiquitin in cargo sorting by ESCRTs

Several ESCRT subunits present ubiquitin-binding domains (UBDs). Some of them,
like HRS and its yeast homologue Vps27 present even several UBDs suggesting an
important role for ubiquitin and ubiquitin-binding during ESCRT mediated sorting
[Hirano 2006, Ren 2010]. The UBDs presented inside ESCRT subunits are presented
in Table 2.2.

It is not surprising that ESCRT-III subunits do not present evidence of ubiquitin
binding as this sub-complex is likely to be involved in membrane deformation and
scission rather that in cargo sorting. See [Henne 2011] for review. While, it has been
proposed that ESCRT-III filaments sequester cargo in the portion of membrane that
will constitute the future bud [Teis 2010], this sequestration could be only physical
with no need for binding between the filaments and the cargo.

Besides, presence of UBDs in every other ESCRT sub-complex may indicate that the
binding of each sub-complex is not only dependent on its interaction with the upstream
complex but also on its interaction with membranes and with ubiquitin-labelled cargo.

Complex	Protein	Ubiquitin-binding domain	References
ESCRT-0	HRS	Double-sided UIM, VHS	(Hirano et al. 2006, Ren et al. 2010)
	Vps27$_{yeast}$	Two tandem UIM, VHS	(Bilodeau et al. 2002)
	HseI$_{yeast}$/STAM1&2$_{mammals}$	UIM, VHS	(Bilodeau et al. 2002, Fisber et al. 2003, Mizuno et al. 2003)
	ESCRT-0 heterodimer	GAT (predicted binding)	(Prag et al. 2007, Ren et al 2009, Mattera et al. 2004, Puertollano et al. 2004, Scott et al. 2004, Shiba et al 2003)
ESCRT-I	Vps23$_{yeast}$/TSG101$_{mammals}$	UEV	(Pornillos et al. 2002b)
	MVB12	Novel UBD	(Shields et al. 2009, Tsunematsu et al. 2010)
ESCRT-II	Vps36$_{yeast}$/EAP45$_{mammals}$	GLUE domain, with inserted NZF in yeast but not in mammals	(Alam et al. 2004, Slagsvold et al. 2005)

TABLE 1.2 – **Ubiquitin binding domains in ESCRT subunits**

UIM stands for ubiquitin-intercating motif. VHS stands for Vps27 HRS STAM. GAT stands for GGAs and TOM. References: [Bilodeau 2002, Pornillos 2002b, ?, Shiba 2003, Mizuno 2003, Mattera 2004, Puertollano 2004, Scott 2004, Alam 2004, Slagsvold 2005, Hirano 2006, Prag 2007, Ren 2009, Shields 2009, Tsunematsu 2010, Ren 2010]

It could also mean that the cargo is transfered from one complex to the next until it is trapped in the bud.

In addition, the presence of several UBDs among one subunit, one sub-complexes or several subcomplex may also enable either to bind several cargos simultaneously, to bind poly-ubiquitinated proteins with increased affinity [Ren 2010] or even to cluster ubiquitinated cargo, which may, as seen in chapter one, adjuvate membrane deformation.

Cargo in MVBs is usually ubiquitinated by Lys63-linked polyubiquitin chains [Galan 1997, Springael 1999, Roth 2000, Huang 2006]. Consistently, the ESCRT complex binds to polyubiquitin chain with more affinity than to monoubiquitin chains (about 50-fold). Only a 2-fold difference has been seen between the binding to Lys63-linked and Lys48-linked chains [Ren 2010], but NZF domain of Vps36 may favor Lys63-linked chains [Alam 2004, Kulathu 2009]. This is consistant with the fact that Lys63-linked chains are important for MVB sorting. See [Lauwers 2010] for review.

Additionally, there are clues for an important role of ubiquitination during virus budding and cytokinesis. See [Shields 2011] for review. For instance ALIX binds, through its V-domain to TSG101 ubiquitin binding domain UEV [Pornillos 2002a, Strack 2003, Martin-Serrano 2003, von Schwedler 2003, Fisher 2007, Lee 2007b]. See

[Odorizzi 2006, Usami 2009] for review. Moreover, if we take a closer look at virus budding, Ubiquitin is a constituent of the virus-like particles (VLP) [Putterman 1990, Ott 1998]. Moreover, Gag proteins containing PPxY motifs can undergo ubiquitination [Ott 1998, Strack 2000]. Also, PTAP binds to the UEV domain of TSG101 which is an Ubiquitin E2 variant domain and it is possible that PTAP recruits E2 enzymes to promote Gag's ubiquitination [Strack 2000]. Moreover, the E3-ubiquitin ligase Nedd4-like has been involved in HIV-1 release [Usami 2009]. Nedd4-1, which also belongs to this family, is likely to mediate HIV-1 thanks to the binding and probably the ubiquitination of ALIX [Sette 2010]. However, if ubiquitin has a very important role in the regulation of cytokinesis [Pohl 2008], information is still missing about the link between ubiquitination processes at the cytoplasmic bridge and the recruitment of ESCRT. Ubiquitination may also play a role in the regulation of some ESCRT subunits themselves. See Table 2.3 for actors and effects of ubiquitination of ESCRT subunits.

Protein	Ubiquitination	Enzymes	Effect
Vps27$_{yeast}$	Monoubiquitination	N.A.	Unknown
HRS$_{mammals}$	Monoubiquitination	DUB: AMSH	Inhibition of ubiquitinated cargo binding
TSG101	N.A.	Mahogunin (E3-enzyme)	Disruption of endosome to lysosome trafficking
	N.A.	Tal (E3-enzyme)	Disruption of MVB biogenesis and virus budding.

TABLE 1.3 – **Ubiquitination of ESCRT subunits**
UIM stands for ubiquitin-intercating motif. VHS stands for Vps27 HRS STAM. References:
[Polo 2002, Stringer 2011, Hoeller 2006, Sierra 2010, Kim 2007a, Amit 2004].

In the case of MVB biogenesis, cargo is deubiquitinated prior to its packaging into ILVs. The activity of de-ubiquitinating enzymes (DUBs), may therefore be important for ubiquitin recycling and for the activity of ubiquitinated ESCRT subunits. In mammals, the ubiquitin isopeptidase AMSH[39] binds to ESCRT-0 (via STAM) and ESCRT-III (via CHMP3) sub-complexes [McCullough 2006, Solomons 2011]. It seems to be responsible for the de-ubiquitination of cargo bound to Lys63-linked poly-ubiquitin chains, and for the de-ubiquitination of HRS [McCullough 2006, Row 2006, Kyuuma 2007, Sierra 2010]. UBPY, another mammalian ubiquitin isopeptidase interacts with ESCRT-0 (via STAM) [Row 2006] and can process both Lys-63 and Lys-48 polyubiquitin chains. In yeast, the DUB Doa4 is responsible for the de-ubiquitination of cargo [Amerik 2000, Swaminathan 1999].
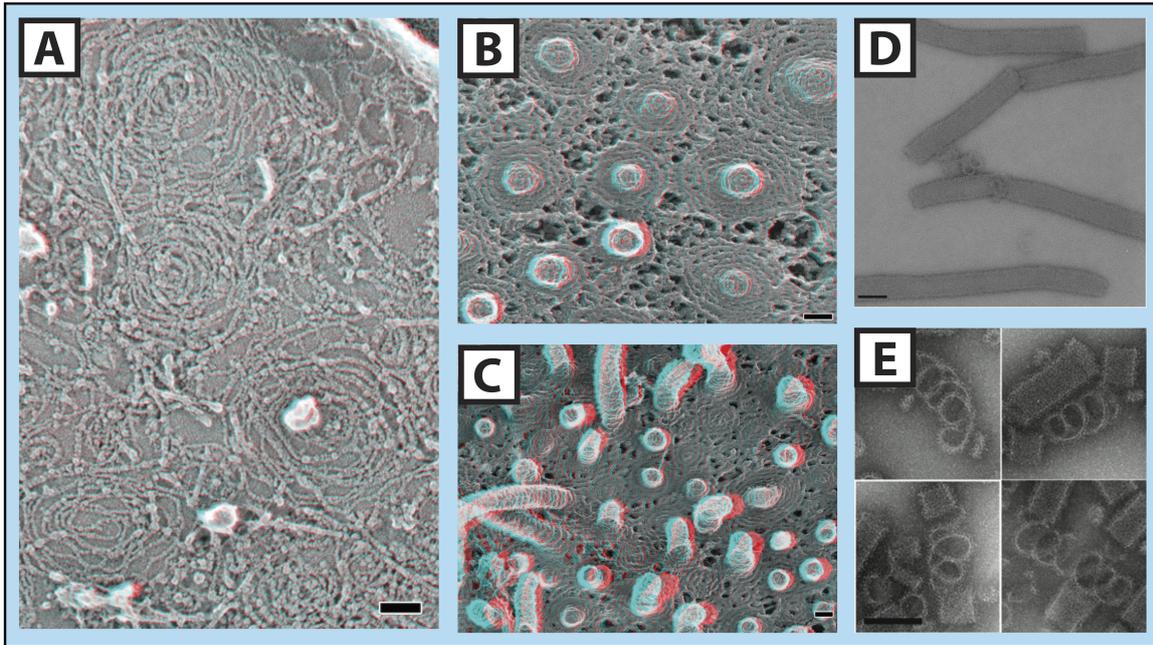
FIGURE 1.12 – **The role of ESCRTs in cellular processes.**
A- Plasma membrane of COS-7 cells expressing Flag-hSnf7-1 (CHMP4A). B and C- Submembranous skeleton of cells co-expressing hSnf7-1 and the catalytically inactive Vps4B(E235Q)-GFP, after detergent extraction. B- View of the "inside" of plasma membrane. C- View of the "outside" of the plasma membrane. A, B and C are adapted from [Hanson 2008]. Anaglyphs of transmission electron microscopy images. Scale bars: 100nm. D- Helical-tubular shaped CHMP2AdeltaC and CHMP3deltaC structures. Negative staining EM. E- Idem in the presence of a DOPS:SOPC bilayer. D and E are adapted from [Lata 2008b]. Scale bars: 100nm.

1.3.2   Membrane deformation and scission driven by ESCRTs

As a reminder, all processes requiring the ESCRT machinery require a three step process:

1. The specific targeting of ESCRT machinery;
2. The deformation of the target membrane to form a nascent vesicle;
3. The scission of the vesicle;

In this part we will review the arguments and models describing how all this ESCRT proteins interplay to achieve the last two steps.

As described previously, physical parameters such as phase separation with its associated line tension can drive spontaneous budding [Hanczyc 2004]. See [Lenz 2009] for review. H. Matsuo and co-workers showed that the presence of the phospholipid lysobisphosphatidic acid (LBPA) can drive the formation if internal vesicles in liposomes [Matsuo 2004]. Even though this assay was done in a simplified system, the result is striking as the LBPA phospholipid is an unconventional lipid, that is exclusively

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39. AMSH stands for associated molecular with Sh3 domain of STAM

detected in late endosomes where it is particularly abundant in the ILVs (about 15% of the total phospholipid fraction) [Kobayashi 1998]. Similarly, K. Trajkovic and co-workers could show in purified endosomes that ceramide enriched ILV-like vesicles could be formed in the absence of ESCRTs and in the presence of sphingolipid ceramide [40] [Trajkovic 2008].

Even if inner vesicles can potentially form without ESCRTs, some of these proteins clearly promote membrane deformation. Indeed CHMP4A and B can polymerize into filaments organized in spirals at the plasma membrane. They are associated with membrane negative curvature revealed by anaglyphs. See pannel A of Figure 2.12. The truncation of CHMP4A inhibitory domain or the co-expression of the dominant negative mutant of Vps4 give rise to membrane buds at the location of CHMP4-A spiral-shaped filaments (observed by trasmission electron microscopy and recreated anaglyphs) [Hanson 2008], suggesting that CHMP4 can promote membrane deformation. See pannels B and C of Figure 2.13.

Recent work *in vitro* strengthen the models for ESCRT-mediated membrane deformation. For instance, CHMP3 can assemble into long helical filaments as it was predicted by its crystal structure [Muziol 2006, Ghazi-Tabatabai 2008]. Associated CHMP2A and CHMP3 can polymerize into filaments as well [Ghazi-Tabatabai 2008]. S. Lata and co-workers could improve the observation of related chimeras: CHMP2 and CHMP3 co-assemble *in vitro* and form helical tubular-shaped structures with a diameter close to the diameter of the neck of viral buds and buds giving ILVs [Lata 2008b]. See pannels D and E of Figure 2.13.

Further *in vitro* studies on liposomes with purified proteins brought convincing arguments for the model of membrane deformation driven by ESCRT proteins. S. Saksena and co-workers, using electron-microscopy, showed that the addition of the four core subunits of the ESCRT-III complex to liposomes triggered the formation of Snf7-dependent (yeast protein for mammalian CHMP4) invaginations of about 40nm in diameter [Saksena 2009b, Wollert 2009] . This study used also fluorescence-spectroscopy-based technique to follow conformational changes in yeast Vps20 and Vps32 upon addition to liposomes revealing important aspects of ESCRT-III complex assembly: 1-The nucleation of Snf7 by Vps20 occurs at the membrane surface after binding of Snf7 on membranes; 2- The conformations of Snf7 oligomers is stabilized or changed by Vps20; 3- These oligomers are capped by Vps24 in a membrane association dependent manner [Saksena 2009b].

In addition, *in-vitro* studies showed that CHMP2 and Vps4 were only required for the recycling of ESCRT proteins, suggesting that CHMP6, CHMP4 and CHMP3 are the key components for membrane deformation and scission among the late ESCRT proteins [Wollert 2009]. Surprisingly, further studies *in vitro* from T. Wollert and J. H. Hurley showed that ESCRT-I and II can also drive membrane budding. The presence of ESCRT-III drives scission of this pre-formed buds [Wollert 2010]. Nevertheless we cannot exclude that CHMP6, CHMP4 and CHMP3 enhance the deformation of the membrane prior to vesicle scission.

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40. Sphingolipid ceramides can be converted by sphingomyelinases into sphingomyelin which can form liquid ordered phases together with cholesterol

The current models described for the CHMP6-CHMP4-CHMP3 mediated abscission, propose that the later shapes of ESCRT-filament are organized in a dome or a cone scaffolding membrane. At the tip of any of these structures, the membranes would be close enough to undergo spontaneous physical fusion/fission [Guizetti 2012].

1.3.3   Models for vesicle generation

Although it is clear that ESCRTs can drive membrane bending and scission of forming vesicles, it is not yet clear how this curvature is generated. Several models have been proposed. See [Hanson 2009] for review and for visual understanding see Figure 2.13.

A first model called the "purse string" model proposed by [Saksena 2009b] suggests a mechanism with a single ring (or several non attached rings) composed of the nucleator CHMP6, the polymerized CHMP4 and the cap CHMP3. The removal of CHMP4 subunits from one end of the polymer would trigger the narrowing of diameter of the ring and a constriction of the membrane inside the ring resulting in budding.

A second model proposed by [Wollert 2009] called the "spiral contriction" model is based on the same concept of the first model, proposing a decrease in the diameter of the membrane portion delimited by ESCRTs, only this time the ESCRT filament form a spiral (or an helix as it has been shown that CHMP4 are associated with certain volume/membrane bending). The decrease in diameter of the surrounded area would result from the centripetal polymerization of the filament. [Hanson 2008])

A third model called the "moving neck" model is based on a spiral/helix-shaped filament [41] which depolymerizes in one extremity (the one towards the vesicle) and polymerizes in the other (the one on the side of the cytoplasm), trapping that way more and more membrane on the side of the vesicle [Hanson 2008].

A fourth model arises from the observations of T. Wollert showing that ESCRT-III proteins are likely recruited to an already ESCRT-I and II-deformed membrane [Wollert 2010]. ESCRT-III would eventually deform the membrane further and ensure constriction by one of the previously described means. The membrane deformation by ESCRT-I and II complexes remains poorly described. Some hypothesis include: 1-membrane bending due to membrane composition and phase separation stabilized by ESCRT-I and II, 2- clustering of specific lipids by interactions with ESCRT-I and II subunits, or 3- clustering of ubiquitinated cargo by these subunits resulting in membrane bending as seen in chapter 1.

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41. The mechanism of this model is logically more compatible with an helix shaped filament rather than a 2D spiral
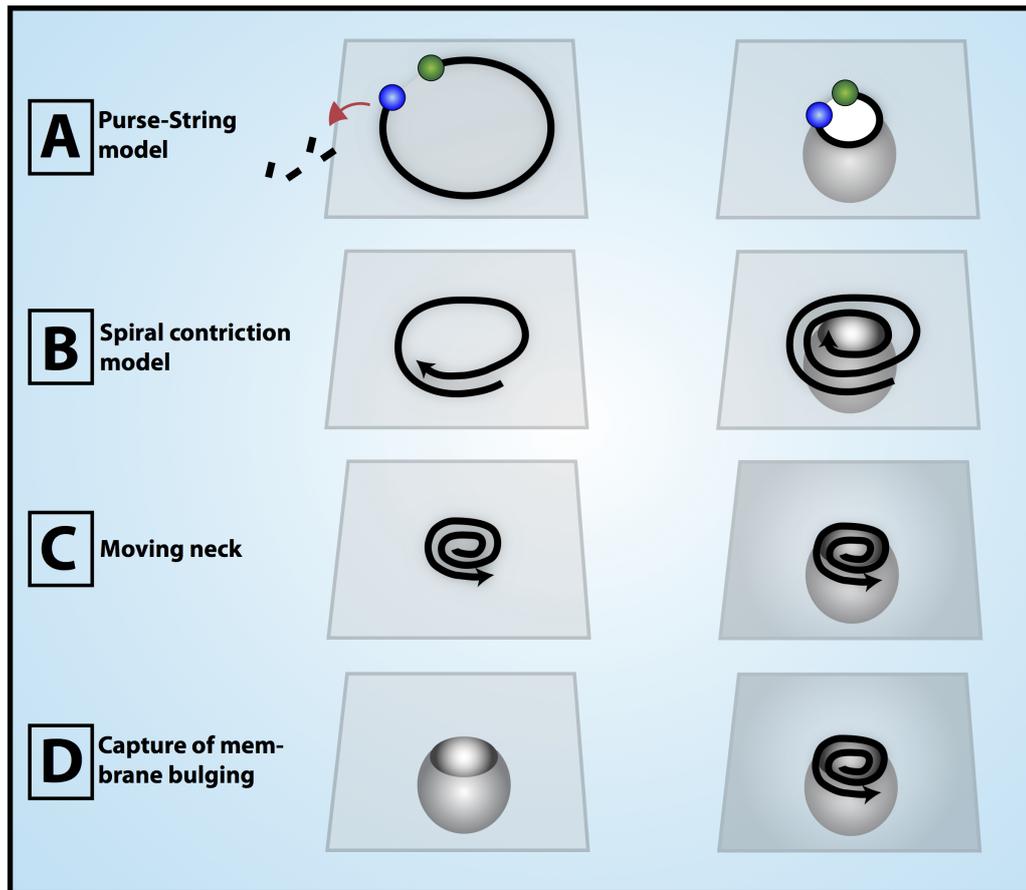
FIGURE 1.13 – **The models for ESCRT-mediated membrane deformation and budding.**
A- Purse-String model proposed by [Saksena 2009b], B- Spiral-contriction model [Wollert 2009], C- Moving neck [Hanson 2008], D- Capture of membrane bulging [Wollert 2010]

1.4 ESCRTs in development and disease

Impairment of the function of ESCRT subunits has been linked to several diseases. They are of different nature probably because of the various functions where ESCRTs have been implicated but also because of the differential requirement of specific subunits in these functions. The following section presents an overview of several diseases to which ESCRTs have been associated and other pathogenic situations that may be modulated by ESCRTs.

ESCRTs in cancer

Two functions of ESCRTs may explain their involvement in cancer: intracellular trafficking and mitosis.

For example, ESCRTs have been implicated in the degradation of protein from the receptor tyrosine kinase (RTKs) family, whose most studied member is EGFR. RTKs are

implicated in the control of several cellular functions including cell growth, proliferation, differentiation of cells in different tissues and organs. See [Hunter 2000, Pawson 2001] for review. EGFR was particularly associated with several types of cancer including mammary carcinomas and gioblastomas. See [Blume-Jensen 2001] for review.

The ESCRT mediated degradation of EGFR and in general of all RTKs may have a strong impact in the regulation of the pathways downstream of these proteins. See [Saksena 2007, Hurley 2006, Williams 2007] for review. Consistently, both ESCRT-I proteins TSG101 and Vps37A were first identified as tumor suppressor genes [Li 1996, Xu 2003]. Actually, reducing the expression of TSG101 in nude mice causes the emergence of metastatic tumors [Li 1996]. This observation is nevertheless contradictory with the fact that TSG101 depletion causes accumulation of two negative regulators of cell cycle, p53 and p21 [Li 2001]. Additionally, the Notch protein seem to be degraded *via* an ESCRT-dependent pathway in drosophila. Moreover, the depletion of the drosophila versions of HRS (ESCRT-0), TSG101(ESCRT-I) and Vps25 (ESCRT-II) causes accumulation of Notch. This accumulation may lead to cell proliferation as showed in the eye disc of drosophila [Thompson 2005, Vaccari 2005, Moberg 2005, Lloyd 2002, Chao 2004, Reynolds-Kenneally 2005].

More clues about the implication of ESCRT-mediated degradation of receptor in cancer is reviewed in [Saksena 2009a]. Another explanation for the implication of ESCRTs in cancer is the requirement of ESCRT-I and III during cytokinesis as described in previous sections. Indeed, the depletion of some ESCRT subunits, the expression of dominant negative constructs or interfering with ALIX-binding site in ESCRT-III subunits lead to the accumulation of multinucleated cells [Dukes 2008, Morita 2007b, Carlton 2008].

RTKs have been also implicated in other diseases such as diabetes, immunodeficiencies and cardiovascular diseases.

ESCRTs in neurodegeneration

ESCRTs role at the level of MVBs has also been associated with other diseases such as a variety of neurodegenerative diseases. Most particularly, we discussed previously about the association between a rare form of an autosomal dominant fronto-temporal dementia (FTD) [42] found in a Danish family and a splice site mutation in the CHMP2B gene [Skibinski 2005, Momeni 2006]. Similarly, mutations in CHMP2B have been observed in some patients with amyotrophic lateral sclerosis. Inclusions in the brain of patients with FTD3 are positive for p62 suggesting a possible implication of autophagy in these diseases by impairement of autophagy-dependent degration of protein aggregates [Talbot 2006]. In addition, recent studies showed that vps24/CHMP3 was required for efficient autophagy-dependent clearance of huntingtin aggregates in neuronal cells [Filimonenko 2007]. These aggregates are associated with Huntington disease although their implication in the disease in not clear yet. ESCRT binding partners like spastin or mahogunin, have been implicated in other neurodegenerative disease although the direct implication of ESCRT has not been studied.

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42. FTD represents the second most widespread form of dementia in presenile patients after Alzheimer's disease [Ratnavalli 2002]

ESCRTs in pathogen infection

Given the implication of ESCRTs in the assembly, budding and release of multiple viruses, it was expected that these proteins would play a major role in viral infection and proliferation. Their implication in HIV-1 which was presented in previous sections has clearly a strong impact for human health. Besides viruses, recent work has shown that ESCRTs may inhibit the proliferation of some intracellular bacteria such as *Mycobacterium fortuitum* [Philips 2008] which is a bacteria closely related to *Mycobacterium tuberculosis*, responsible for tuberculosis.

ESCRTs in other diseases

Mutations in CHMP4 protein have been associated to some forms of cataract [Peterson 2010].

ESCRT proteins have also been indirectly associated with other diseases (see [Saksena 2009a] for review) especially in renal disease FSGS (focal segmental glomerusclerosis) through the mutation of the TSG101 binding partner CD2AP [Kim 2003].

Interestingly, it is likely that ESCRT are implicated in many other diseases through their role in degradation. For example, they are implicated in the degradation of ion channels such as ENaC (epithelium sodium channel). A mutation or a missregulation in the number of channels at the plasma membrane have been associated with several diseases including renal diseases, cardiovascular morbidity and hypertension [Botero-Velez 1994, Stutts 1995, Chang 1996, Saksena 2009a]

The plasma membrane integrity: a fight between threats and repair

As the most exposed structure in the cell, the plasma membrane is prone to recurrent physical, chemical and biological aggressions. These aggressions perturb the plasma membrane permeability and lead to cell death when not reverted. Besides, the plasma membrane wounding can also contribute to physiological adaptations such as the proliferation of cardiac cells upon the release of FGF following plasma membrane wounding.

Several mechanisms for plasma membrane repair have been described. The nature of mechanisms involved in each episode of plasma membrane wounding probably depends on the characteristics of the wound (size, nature...) and the cellular context. In this chapter, we will try to answer to the following questions: What events can lead to the disruption of the plasma membrane? How often does this happen? Are there zones particularly sensitive to disruption? How are disruptions reverted to avoid the spilling out of the cytoplasm?

Several mechanisms including patching the wounded membrane, lowering membrane tension and internalizing pore-forming toxins causing membrane wounding have been implicated in membrane healing. In this chapter I will give an overview of the factors (intrinsic and extrinsic) that constitute threats for membranes and most particularly to the plasma membrane. An overview of the mechanisms of plasma membrane repair will be presented.

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Tables

2.1   Threats for the plasma membrane: menace for the cell, tools for the scientist.

The threats for the plasma membrane are of very different natures: physical, chemical or biological. These threats can come from the activity of the cell itself, from the activity of the tissue to which the cell belongs or from the environment surrounding the cell. Most of these threats and the capacity of the cell membrane to be healed have been exploited by biologists to introduce in the cell cytoplasm molecules that do not normally pass through the cell membrane. Other sources of damage are used for other bioclinical assays such as radiography were the membrane damage is not an aim but only a hazardous side-effect.

The intensity of the threat is crucial in natural conditions and in experimental conditions as a very intense threat of any nature will trigger cell death.

Organ	Type of mechanical force	Experimental procedure	Principal cells stressed	Proportion of cells wounded	References
Skeletal muscle	Aperiodic, highly variable intensity: shear, stretch	Rats exercised intensively by downhill running and entry of the rat's albumin detection(McNeil and Khakee, 1992)	Skeletal muscle cells (myocytes)	3-20%	(McNeil and Khakee, 1992)
Cardial muscle	Cyclic: shear, stretch	Exercice induced contractions and and injection of beta-adrenergic agonist isoprotereol	Cardiac myocytes	25%	(Clarke et al., 1995; Fischer et al., 1997)
Gastrointestinal tract	Cyclic: shear, stretch		Epithelial cells, smooth muscle cells	Epithelial cells wounded (not measured)	(McNeil and Ito, 1989)
Vascular (conducting)	Constant and cyclic: shear		Endothelial cells, smooth muscle cells	6.5% of aortic endothelial cells	(Yu and McNeil, 1992)
Respiratory	Cyclic: stretch		Epithelial cells, endothelial cells, smooth muscle cells	2- 30% of alveolar cells in mechanically ventilated lung	(Gajic et al., 2003; Mendez et al., 2004; Doerr et al., 2005)
Skin	Aperiodic, highly variable intensity: shear, stretch, compression	Locomotion	Epidermal cells, Fibroblasts, etc.	6-15%	(McNeil and Ito, 1990)

TABLE 2.1 – **Cell plasma membrane wounding by physiological mechanical constraints**

This table table was adapted from [McNeil 2003]. References: [McNeil 1992, Clarke 1995, Fischer 1997, McNeil 1989a, Yu 1992, Gajic 2003, Mendez 2004, Doerr 2005, McNeil 1990]

2.1.1 Cell plasma membrane wounding by physiological mechanical constraints

Plasma membrane is naturally and frequently prone to disruptions when placed in a mechanically stressful environment. Some of the most damaged tissues are of course the ones that are exposed to strongest and longest physical constraints. Those are mainly the tissues presenting a high contractile activity such as the skeletal muscle myocytes [McNeil 1992], the cardiac myocytes [Clarke 1995, Fischer 1997], epithelial and smooth muscle cells from the gastrointestinal tract [McNeil 1989a] and, in a smaller proportion, endothelial and smooth muscle cells from the vascular systems [Yu 1992]. Some of these cells are also submitted to shear stress, especially in the gastrointestinal tract and the vascular systems. See Table 3.1. Other tissues that do not have an intrinsic contractile activity are subject to mechanical stress due to the function of the tissue where they belong. It is the case of cells in lungs were endothelial, epithelial and smooth muscle cells present wounding due to stretching caused by ventilation (See Table 3.1).

To perform *in vivo* tests, most of this studies used perfusion of fluorescent and non-permeable dyes such as PDx or Propidium Iodide or the animal's endogenous albumin to detect wound-repaired cells. See Figure 3.1.

2.1.2 External physical threats

Mechanical threats

The causes for cellular damage can be as evident and unexpected as there are things we can manipulate. P. L. McNeil and S. Ito used daily life threats for the skin (scratching, tape stripping and needle puncture) and they could observe cell membrane damage in all conditions [McNeil 1990].

Other less expected mechanical threats such as sound have to be taken into account. The sound is a mechanical wave generated by the vibration of a liquid or solid material. It propagates as an oscillation of pressure transmitted through a solid, a liquid or a gas medium. The very mechanical nature of the sound waves explain by itself why sound can damage materials depending on their elasticity properties. For our matter, it has been shown that sound can damage the plasma membrane of the hair cells in the inner ear of a lizard perfused with Lucifer Yellow and submitted to strong sound [Mulroy 1998]. From a biotechnological point of view, the technique of increasing membrane permeability with sound (usually ultrasonic frequencies) is called sonoporation or cellular sonication. It has been exploited as a transfection technique to introduce large molecules of DNA. Controlling the exposure to ultrasounds while performing this kind of experiment is necessary to avoid cell death. Sonoporation is being studied as a therapeutic method to deliver DNA [Zolochevska 2012].

Other useful technics in cell biology and neurosciences rely as well in the capacity of the cell to recover from mechanical disruption of its plasma membrane. An example is the use of a micropipette for microinjection or for measurements of membrane potential.
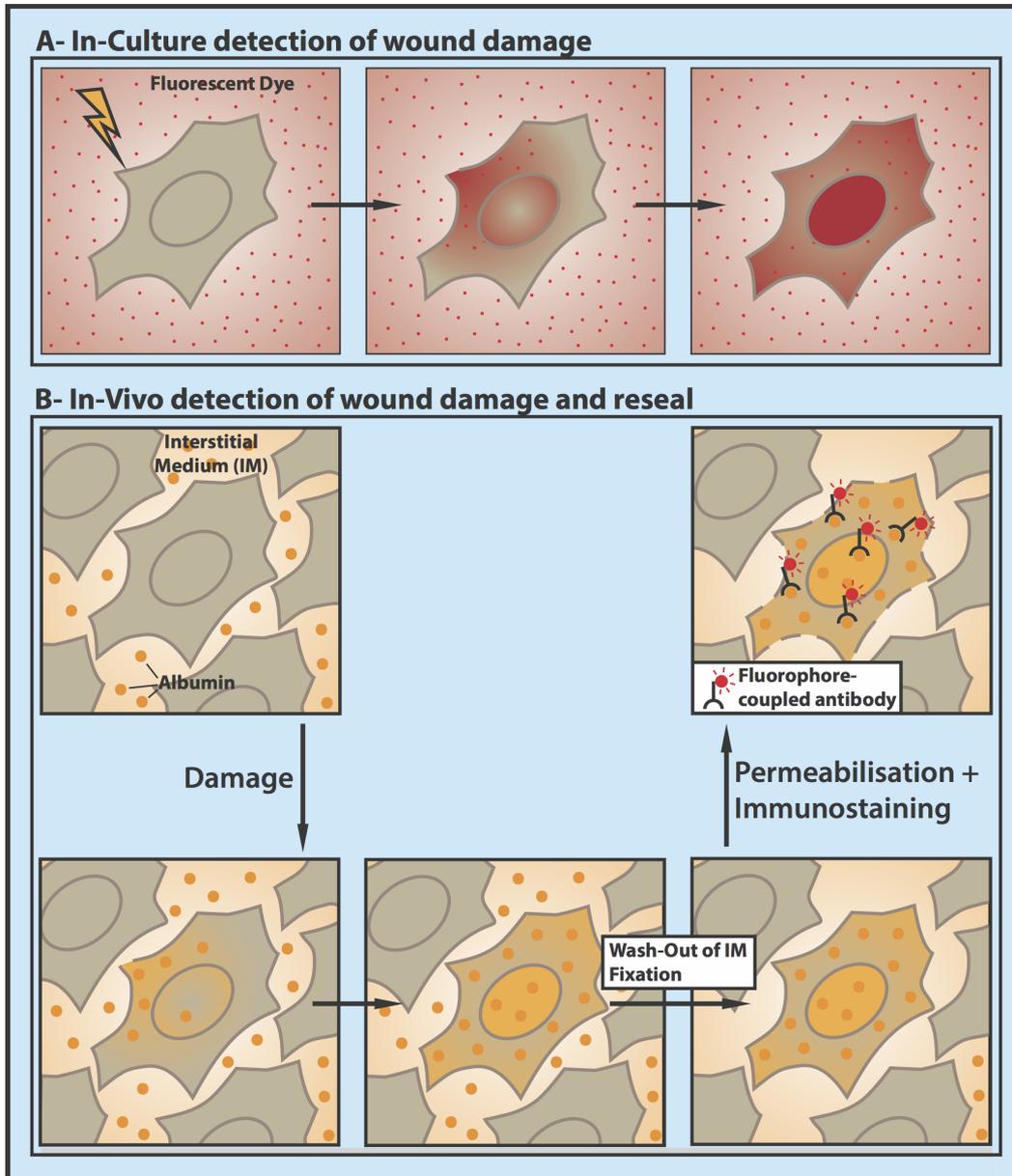
FIGURE 2.1 – **Detecting wound-repaired cells in vivo and in cell culture.**
This figure shows two very common assays used to detect membrane wounding and repair. A- Assay used in cultured cells, where it is possible to add fluorescent dyes to follow and measure the opening and the closure of a membrane wound. B- Assay used to study *in vivo* the occurence of wounds and their resealing. The albumin is naturally present in the interstitial medium. A cell that undergoes wounding and succeeds the resealing of its membrane will capture albumin from the interstitial medium. This assay is based on the post-fixation immunofluorescence or immunohistochemistry detection of albumin inside the cells of a tissue.

Electric fields and electroporation

Electroporation is a powerful tool to increase cell membrane permeability and to introduce exogenous molecules into the cytoplasm. It is used in the medical field to introduce chemotherapeutic agents (process called electrochemotherapy) or DNA (process called electrotransfer). In the biology research field, it is widely used to introduce large molecules of DNA and RNA, proteins or transfecting cells that resist to transfection reagents. It has also been used in an irreversible fashion to provoke cell death while releasing intact molecules from cell cytoplasm [Phillips 2012]. Most of these techniques rely on the capacity of the cell to repair its plasma membrane.

Electromagnetic waves threats and tools to image and create accute membrane wounds

Electromagnetic waves are present in our daily life especially with visible light, which we use for most of our daily tasks. See the electromagnetic spectrum in Figure 3.2.

Visible light wavelengths range from 380 nm to 740 nm. This radiation as we use it every day can rarely be a threat because of its low intensity and its non-coherent nature. Nevertheless in many experimental and clinical conditions, intense illumination [1] or lasers have very useful applications in fluorescence microscopy or clinical surgery. The deleterious power of light on cellular membranes, and especially of lasers, has been exploited in the field of cell membrane repair to reproduce acute holes of sizes compatible with reseal and similar to the ones occurring *in vivo*.
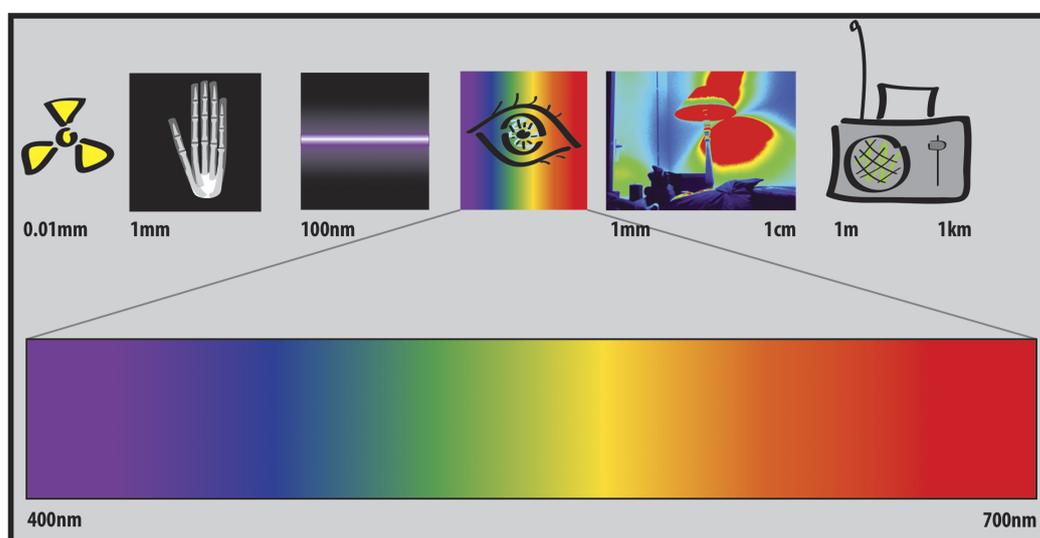
FIGURE 2.2 – **The Electromagnetic wave spectrum.**

Infrared is another example of electromagnetic wave that is harmless in the daily life of an organism and its cells. Infrared radiation has higher wavelength that the

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1. Fluorescence microscopy uses currently mercury lamps for intense illumination
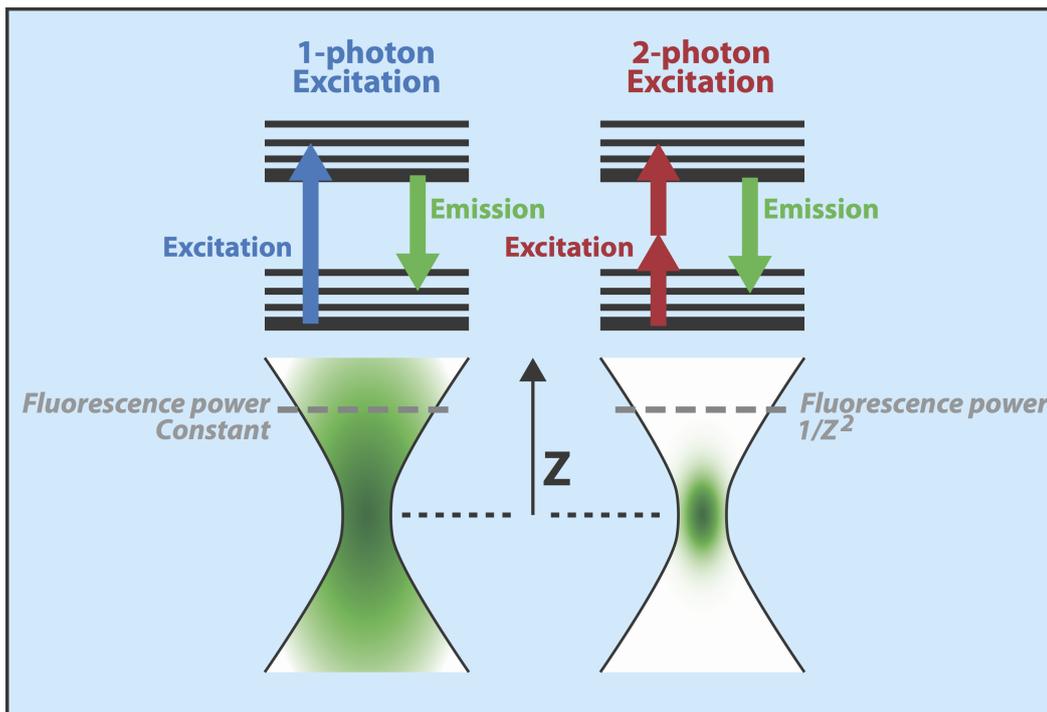
FIGURE 2.3 – **Mono and multi-photon lasers**

visible light. It extends from 740 nm to 300 μm and has relatively low energy. They represent more than 50 per cent of the solar energy arriving in the planet. Infrared is commonly associated with heat because at room temperature, objects naturally emit radiations in the infrared range of wavelength. They have been exploited for imaging, tracking and heating among many other high technology fields like communications. Living beings are thus constantly exposed to infrared radiation which remains mostly harmless. In the biology field, the infrared has been implemented quite recently in the field of imaging living organisms by using the 2-photon excitation principle. This principle first proposed by Maria Goeppert Mayer and then demonstrated by Isaac Abella, is based on the idea that two-photon of comparable energy and lower than the one need for one-photon excitation, can excite a fluorescent molecule in one quantum event. See Figure 3.3. Approximately half of the energy necessary to excite the fluorophore is contained in each photon. This principle is applicable to multi-photon excitation. Multi-photon microscopy has the advantage to penetrate deeper in thick samples and the extremely useful advantage to reduce phototoxicity by ensuring optical sectioning effect at the excitation level. The excitation takes place at the focus point where the pulsed laser beams are more concentrated. This principle was useful for experiments performed in this work, which will be presented later on.

The ultraviolet radiation is, contrary to the infrared, higher in energy than the visible light. These electromagnetic waves range 100 nm to 380 nm wavelength. They represent about 3 per cent of the sun radiation, and their harm for tissues have been of major interest because of their correlation with cancer. Their capacity to produce

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free radicals constitute also a menace for the cell as free radicals have been shown to react with all biological molecules *in vitro* and it is probably the case *in vivo* as well [Evans 1999]. From a laboratory point of view, UV laser equipped microscopes have been powerful tools to make wounds because of the high energy carried by UV-light. They are more toxic but less costly than multi-photon systems and have been of great utility in this work.

2.1.3 Chemical threats

Free Radicals

Free radicals are also non negligible threats for biological membranes. Indeed, their production may be triggered by outside factors such as UV-light, but they are also produced by intracellular reactions: 1- by mitochondrial electron transport in conditions where the respiratory chain is strongly reduced, 2- by cytoplasmic proteins (such as xanthine dehydrogenase), 3- by membrane enzymes (such as lipoxygenase or cycloxygenase), 4- by peroxisomes. See [Martinez-Cayuela 1995] for review. Free radicals can trigger protein oxidation but also lipid oxidation triggering membrane erosion. See [Evans 1999] for review.

Amphipatic molecules
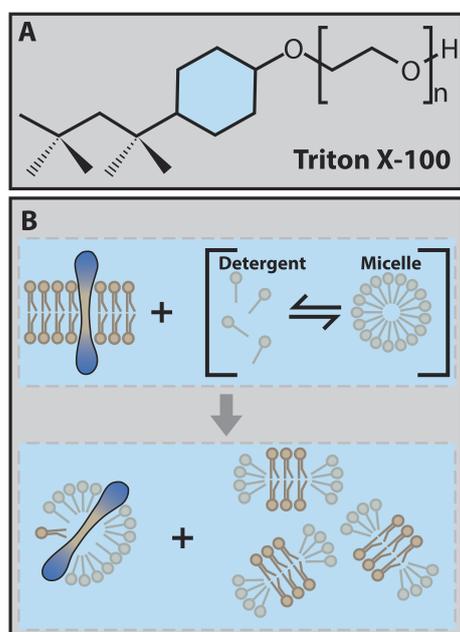
FIGURE 2.4 – **Effect of detergents in membranes and membrane proteins.** Based on [Alberts 2002].

Some molecules produced by living organisms or synthetically have amphipatic characteristics, meaning that they have both hydrophilic and lipophilic properties. Because of their polar head, of their hydrophobic tail, and of their linear structure, these molecules, commonly called detergents, are able to solubilize proteins and phospholipids from the membranes by forming lipid-detergent micelles. See Figure 3.4 for visual comprehension of lipid and protein solubilization by amphipatic molecules.

There exist detergents with different strengths and, therefore, with differential capabilities of solubilizing lipids and proteins. The Sodium-Dodecyl-Sulfate (SDS), for example, is a powerful detergent thanks to its ionic nature. It does not only solubilize proteins and lipids but can also unfold proteins by interacting with their hydrophobic domains.

There exist also milder non-ionic detergents that will not unfold proteins. A commonly used mild synthetic detergent is Triton X-100. At very low doses, it is possible to create a limited number of holes in the membranes. This property is very useful for immunolabelling of intracellular components but has also been used seldomly to study membrane repair [Calvello 2011].

Other natural molecules with amphiphatic properties such as saponins are also commonly used for the same permeabilization purposes. Saponins are very diverse and complex glycosides [2] produced by some plants, plankton and sea animals such as sponges or sea cucumber. See [Francis 2002] for review. Their name derives historically because of their ability to make stable soap-like foams in aqueous solutions. They are used in medicine and biology for their detergent-like properties. Saponins increase cell membrane permeability by making insoluble complexes with cholesterol thanks to the affinity of their aglycone [3] moiety to the membrane sterols [Bangham 1962]. A consequence of this property is that saponins will preferentially permeabilize cholesterol rich membranes as shown by [Gogelein 1984]. Therefore, less quantity is needed to permeabilize the plasma membrane than to permeabilize membranes with low cholesterol such as the nuclear envelope. This characteristic has made them good candidates to study plasma membrane repair in response to non-proteic pores [Cai 2009, Zhu 2011]. Digitonin has also been used to permeabilize cell membranes, and specially cholesterol rich membranes. It is produced by *Digitalis purpurea*. It does not belong to the Saponins group but has similar chemical characteristics as it is also a glycoside. Its aglycone is a steroid called digitogenin. Both Saponin and Digitonin are believed to form micelle-like structures inside the lipid-bilayer, and forming, therefore, a pore rather than solubilizing proteins and lipids as a regular detergent like Triton does [Gogelein 1984]. Both molecules have been useful to study plasma membrane repair in this work.

2.1.4 The Proteic threats

The pore forming toxins

We saw previously that some organisms, eukaryotic or prokaryotic, are capable of secreting non-proteic molecules capable of making pores in the plasma membrane. They probably constitute a weapon to defend themselves against other organisms or to take advantage of them. Other organisms have selected secreted proteins for the same purpose. They represent about 30% of known bacterial toxins [Alouf 1999], but they are also produced by higher organisms, for example, sea anemones [Bernheimer 1976].

The pore forming toxins are, as their name indicates, proteins capable of making lesions in the plasma membrane by assembling into pore-like structures. Many of them contain beta-sheets and undergo oligomerization.

Both channel proteins and pore forming toxins (PFTs) are capable of creating communicating passage between the cytoplasm and the cell environment. However, if

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2. A glycoside is a molecule form by the binding of a sugar on a non-carbohydrate moiety which is very often a small organic molecule.

3. Aglycone stands for the non-sugar group of a glycoside

cells are able to control the opening and the closure of their channel proteins, they have usually only one way to close pore forming toxins and that is by removing them from the plasma membrane.

The PFTs can be classified in two main groups according to their structure and, therefore, according to the way they cross the cell membranes. These two groups are: 1- the alpha-PFTs which are alpha-helical proteins; 2- the beta-PFTs which are beta-barrel proteins. See [Iacovache 2008] for review.

Among the beta-PFTs we find the most common PFTs involved in human health: 1- cholesterol-dependent cytolysins (and among them Streptolysin-O [4] that has largely been used in laboratories for plasma membrane repair studies); 2- PFTs produced by *Staphylococcus aureus*[5]; 3- AB type toxins (and among them anthrax toxin) [6]. See [Iacovache 2008] for review.

Importantly, prior their insertion into membranes, pore-forming toxins attach to specific receptors that can be lipids or proteins. For example, Streptolysin-O binds to cholesterol prior to its insertion into the membrane and forms a pore of about 30 nm in diameter [Tweten 2005]. For further examples of PFTs receptors see review [Lafont 2004]. Note that PFTs are not specific to plasma membrane disruption. Some PFTs have the capacity to rather form pores in intracellular membranes. For example, the folding of listeriolysin O into a pore is pH dependent. This toxin is thus inactive at the plasma membrane and becomes active in endosomes [Geoffroy 1987]. Another example is the pore-forming toxin VacA which is produced by *Helicobacter Pylori* and which causes permeabilization of the outer membrane of mitochondria, and promotes apoptosis [Galmiche 2000, Willhite 2004, Willhite 2003].

Another example is the complement. It comprises a number of circulating or membrane proteins of the blood which act as adjuvants of the immune system by inducing non-specific or antibody-driven cytolysis. They are inactive at steady state and are activated by cascades of cleavage. All cascades activating the complement pathway lead to the formation of a transmembrane channel that changes cell membrane permeability aiming at cytolysis. Certain cells may be able to revert the attack of the complement through mechanisms of plasma membrane repair that resemble mechanisms employed against pore-forming toxins [Morgan 1987].

Endogenous and disfunctional proteins

In certain cases, plasma membrane damage can be due to endogenous proteins. For example, some neurodegenerative diseases may be caused by changes in the permeability of neurons plasma membrane, this time through defective proteins that aggregate and form ring-shaped intermediate structures. This ring-shaped structures have been observed both *in vitro* and on lipid bilayers for Amyloid-beta [Hafner 2001,

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4. Streptolysins are streptococcal hemolytic proteins that are secreted (exotoxins)

5. PFTs from *Staphylococcus aureus* include enterotoxin which is produced by some strains and causes food poisoning

6. Anthrax toxin is produced by *Bacillus anthracis* and is the cause of anthrax disease, which is lethal for most of its forms.

Lin 2001, Klug 2003, Lashuel 2003, Lashuel 2006], which is the main constituent of the extracellular amyloid plaques characteristic of Alzheimer's disease [Glenner 1984]. Mutated forms of Amyloid-beta that are associated with familiar Alzheimer's disease promote annular protofibrils *in vitro* [Nilsberth 2001, Lashuel 2002b, Lashuel 2002a]. Interestingly, Arispe and colleagues showed that Amyloid-beta form calcium channels in lipid bilayers, which are at least partially responsible for the disease [Arispe 1993, Arispe 1994]. This observation has been reproduced in many other membrane models.

Similarly, mutated alpha-synuclein (the main component of the intraneuronal Lewis Bodies in all the patients with Parkinson's disease) form annular and tubular structures (as it is also the case for wild type proteins after long incubations) [Conway 2000, Ding 2002, Lashuel 2002b, Lashuel 2002a]. alpha-synuclein's annular structures have morphological and dimensional properties close to the ones made by protein toxins such as hemolysin and aerolysin [Valeva 1997, Orlova 2000, Wallace 2000] and bind to membranes causing permeability for small compounds of less that 2.5 nm in hygrodynamic radius [Volles 2001, Volles 2003].
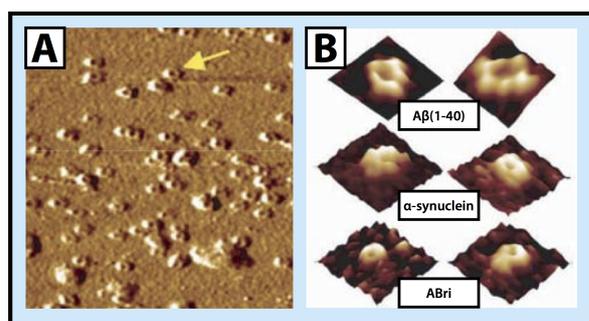
FIGURE 2.5 – **Endogenous proteins adopting pore conformation**

Pore-shaped structures reconstructed in lipid bilayers imaged by AFM (Atomic Force Microscopy). A- Amyloid-beta. Adapted from [Lin 2001]; B- Amyloid-beta, alpha-synuclein and Abri. Adapted from [Quist 2005].

Other examples include annular structures formed *in vitro* by ABri [El-Agnaf 2001]. ABri is the main component of amyloid deposits found in the brain of patients with familial British dementia, although the toxicity of these annular protofibrils *in vivo* has not been characterized [Srinivasan 2004]. Also, the Superoxide dismutase-type 1 (SOD1)[7] form mainly spherical and annular aggregates *in vitro*, with a more rapid aggregation for SOD1 containing pathogenic mutations [Chung 2003, Ray 2004]. Interestingly, metal depletion or oxidation of SOD1 also promote the assembly of annular structures in both wild-type and mutant versions of the protein [Chung 2003, Elam 2003, Ray 2004]. This observation points out that oxidative processes such as the ones promoted by free radicals, may not only damage membranes by oxidizing lipids but also by oxidizing proteins and causing their improper folding.

Moreover, another example is an abnormal form of the prion protein (PrP), PrP-Sc, which causes prion diseases such as Creuzfeldt-Jacob disease, Gertsmann-Straussler syndrome and fatal familial insomnia in humans. PrP-Sc is richer in beta-sheet structures than the normal protein. A truncated version of PrP forms also multimeric spherical and annular structures *in vitro* [Sokolowski 2003]. PrP can associate with the plasma

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7. SOD-1 has been implicated in amyotrophic lateral sclerosis [Rosen 1993, Brown 1997, Shibata 1996].

membrane [Lehmann 1996]. Furthermore, several peptides deriving from PrP also interact with membranes and form ion channels [Lin 1997, Kourie 2000, Bahadi 2003].

Abnormal proteins capable of perturbing the permeability of the plasma membrane are also found in diseases other that neurodegenerative diseases such as renal diseases. For example, abnormal opening of the epithelial sodium channel (ENaC) is linked to renal diseases such as cystic Liddle's syndrome, fibrosis and pseudohypoaldosteronism type I [Stutts 1995, Chang 1996, Botero-Velez 1994].

These pathological proteins present other common characteristics with pore-forming toxins as in both cases the assembly into a ring-shaped amyloid is promoted by lipids [Kayed 2009] and dependent on cholesterol [Arispe 2002, Curtain 2003]. Altogether, these examples suggest that the understanding and control of cellular mechanisms involved in the elimination of pore-forming molecules are crucial to understand and potentially control many genetic and infectious diseases.

In conclusion, the threats for biological membranes are very diverse and ubiquitous. It is important to point the fact that most of these assaults such as mechanical damage, electrical current, electromagnetic waves, free radicals or pore forming toxins are likely to damage the plasma membrane but also organelle membranes. In the next section, we will discuss the mechanisms of repair for a membrane that cannot be eliminated: the plasma membrane. We will further discuss in Chapter 4 the mechanisms that the cell employs to deal with damaged intracellular membranes, which are elimination mechanisms instead of repair mechanisms.

2.1.5 Preventing membrane disruption: the factors of membrane vulnerability or resistance to damage

The cell wall

If all cells in the living kingdom share the characteristic of being bound by a membrane, many type of cells present reinforcement for the plasma membrane boundary by one or even two cell walls. Cells belonging to certain species of Plants, Algae, Fungi, Yeast and Bacteria possess a cell wall. This wall can present very different compositions from one organism to the other. These walls are potentially flexible, but they are kept rigid by the pressure exerted by the cell underneath. This rigidity in turn, protects the cell from mechanical injuries.

John Howland describes the cell wall as following[McNeil 2003]: "Think of the cell wall as a wicker basket in which a balloon has been inflated so that it exerts pressure from the inside. Such a basket is very rigid and resistant to mechanical damage. Thus does the prokaryote cell (and eukaryotic cell that possesses a cell wall) gain strength from a flexible plasma membrane pressing against a rigid cell wall".

Tissue and plasma membrane architecture

The architecture of a tissue is important for protection against membrane disruption, and organisms have adapted so that the most exposed tissues are better wrapped against shocks. It is the case of palms and feet which are protected by layers of fat and connective tissue which may play a role of "shock-absorbers" [McNeil 2003].

At the cell level, some proteins like keratins are required for the maintenance of tissues with strong mechanical constrains. For example, the disruption of K-14 (prominent keratinocyte keratin) leads to an increased skin and epithelium fragility [Chan 1994, Rugg 1994]. Impairment of other keratins result also in more fragile skin and of several other tissues like skeletal and cardiac muscle, tongue and oral mucosa, cornea, rectum and placenta as reviewed in [Coulombe 2002]. This reveals a striking importance of the intermediate filaments in the maintenance of membrane integrity. Besides, mutations in Plectin (protein which links intermediate filaments with actin-containing cell cortex) induce dystrophy in cardiac and skeletal muscle [Smith 1996].

Furthermore, the Dystrophin-Glycoprotein complex, which may make a link between the cytoskeleton and extracellular matrix in muscle cells, has been associated with increased disruption of the sarcolemma [Menke 1991, Petrof 1993, Kabaeva 2011]. The mutation of Dystrophin is also linked with 6 genetic muscular dystrophies [Straub 1997] and most particular Duchenne and Becker's muscular dystrophies. Mutations in sarcoglycans have been implicated in limb-girdle muscular dystrophy and mutations in alpha2-laminin have been shown to cause congenital muscular dystrophy [McNeil 2003]. Additionally, Caveolin-3 seems to play a major role in membrane integrity and resistance to damage. A disfunctional Caveolin-3 causes several forms of skeletal muscle disorders. One case of cardiomyopathy has also been described. See [Gazzerro 2010] for review. Caveolin-3 associates to the Dystrophin-Glycoprotein complex through beta-dystroglycan which is an integral membrane subunit of this complex[8] [Song 1996, Sotgia 2000]. Interestingly, Dystrophin and Caveolin-3 compete for binding to beta-dystroglycan *via* its PPXY motif [Rosa 1996, Jung 1995, Rentschler 1999, Huang 2000, Sotgia 2000]. PPXY is a motif found among the late-domains that exist in several virus genomes. As a reminder, late-domains are necessary for the recruitment of ESCRT machineries during virus budding (see Chapter 2).

Changes in Caveolin-3 expression lead to the disruption of the Dystrophin-Glycoprotein complex [Sotgia 2000, Herrmann 2000]. Intriguingly, it looks like both the depletion and the over-expression of Caveolin-3 affect the formation of the Dystrophin-Glycoprotein complex, although we cannot exclude a dominant negative effect of Caveolin-3 over-expression, a very strong competition for binding to beta-dystroglycan, or an effect of the downregulation of the complex observed by F. Galbiati and co-workers [Galbiati 2000a]. Caveolin-3 null mice have abnormal distribution of the Dystrophin-Glycoprotein complex and present t-tubule organization abnormalities [Galbiati 2001]. It is then very likely that Caveolin-3 participates to the normal development and architecture of muscle fibers. Its role in membrane disruption seems to be rather protective.

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8. It seems that Caveolin-3 interacts with this complex but with a weaker affinity than the one between Dystrophin and Glycoproteins. Moreover, Caveolin-3 is not necessary for the formation of Dystrophin-Glycoprotein complex [Crosbie 1998]

Caveolin-3 is also required for the earlier described "caveolae membrane reservoir", and in that sense, this protein may play a crucial role in the resistance to membrane stretching caused by changes in osmotic pressure, or by pure mechanical stress. However, it has been shown that Caveolin-3 interacts with Dysferlin [Matsuda 2001], which is believed to be involved in repair rather than in architecture of the plasma membrane, as we will see later in this chapter.

The membrane lipid composition and its resistance to damage

The concentration of certain lipids confer to the membranes a certain risk to be the target of lipid specific threats. The best examples are the pore forming molecules and pore-shaped defective proteins which bind preferentially to cholesterol rich membranes. The more cholesterol-rich is the membrane, the more it will be threatened by these molecules. As described previously, Saponins and Digitonin have a special affinity for cholesterol [Gogelein 1984]. Many bacterial pore forming toxins, like Streptolysin-O, have also special affinity for cholesterol. Other, like Aerolysin or Lysenin, have a special affinity for lipid raft components such as GPI-anchored proteins or Sphigomyelin respectively. These molecules are lipid raft components and cholesterol is necessary for the binding of these toxins. See [Lafont 2004, Lafont 2005] for review.

2.2 Membrane repair mechanisms

2.2.1 Detecting and responding to plasma membrane wounding

It has been shown that the presence of Ca^{2+} and the exocytosis of vesicles towards the wound are necessary for the cell to patch large wounds and reseal the membrane [Bi 1995, Miyake 1995, Steinhardt 1994, Terasaki 1997, McNeil 2001]. Calcium is also necessary for cell reaction to pore-forming toxins. In addition of Ca^{2+} dependent exocytosis, Ca^{2+} dependent endocytosis is required for the removal of pore-forming toxins from the plasma membrane [Idone 2008b]. It is therefore required for the survival of cells undergoing wounds of about 1 μm or more [Walev 2001] but is not required to seal holes smaller than 0.2 μm, which are believed to seal spontaneously and extremely fast [Muller 2003, Djuzenova 1996, Shirakashi 2002]. Thus, Ca^{2+} is likely to be a universal mechanism for plasma membrane injury sensing.

Additionally, the exposure of proteins to the extracellular medium, which is more oxidative compared to the cytoplasm, has been shown to be important for membrane repair. MG53[9] is a protein involved in sarcolemma and C2C12 (mouse myoblast cell line) plasma membrane repair. Its oligomerization and its translocation to the wound site are stronger and faster in the presence of thimerosal (oxidizing agent), and is inhibited when cells are incubated in a reduced extracellular medium [Cai 2009, Hwang 2011]. Finally, PTRF[10], which is necessary for MG53 recruitment, has been proposed to sense wounds by binding to exposed cholesterol [Zhu 2011].

Other mechanisms involved in plasma membrane wounding detection are the molecules that escape from the cell through the wound. Rather than a signal for plasma membrane repair, this constitutes an important signal at the level of a tissue. The most studied signal is FGF2[11], which is released upon membrane wounding of muscle cells [Clarke 1993]. Consistently, patients with Duchenne muscular dystrophy present higher blood levels of FGF2 than control patients [D'Amore 1994]. High blood levels of FGF2 are also found when the activity of ex-vivo rat hearts is stimulated with isopeterenol [Clarke 1995]. FGF2 release associated with cardiac myocytes membrane injury (favored by electrical stimulation) is correlated with an increased growth. This suggests that at a tissue level, the release of FGF2 stimulates cell growth, probably in an attempt to replace injured or potentially dying cells [Kaye 1996].

High levels of creatine-kinase and lactate dehydrogenase can also be found in the muscles from patients with Duchenne, Becker or limb girdle muscular dystrophies. See [McNally 2007] for review.

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9. MG53 stands for mitsugumin-53
10. PTRF stands for Polymerase Transcriptase Release Factor
11. FGF2 stands for Fibroblast Growth Factor 2

2.2.2 Self sealing: experiments in simple reconstitution systems
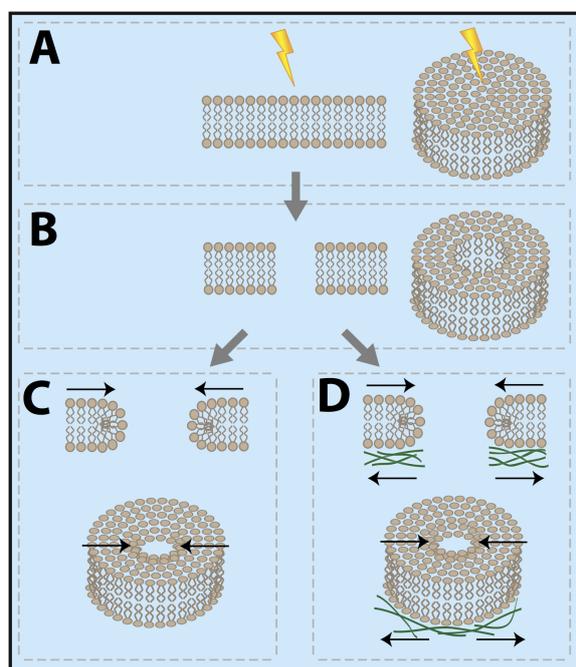
FIGURE 2.6 – **Resealing membrane holes in simple of complex systems.**

A- A healthy membrane. B- A wounded membrane at very early stages. C- In a simple system such a liposome made of phospholipids only, a line tension force will favor the hole closure. D- In a more complex system like a cell where membrane interact with cytoskeleton, a tension force will oppose to the line tension force which will oppose to the closure of the hole.

A disorder imposed in lipid packing, including holes, sort of defines an interface between a disordered domain and the rest/ordered membrane. This interface gives rise to a certain "line tension" which will spontaneously tend to decrease and, therefore, to trigger pore closure, more or less rapidly depending on the viscosity of the medium [Sandre 1999].

If a tension is imposed to the liposome by aspiration or osmotic shock, this tension will oppose to line tension and, therefore, to membrane reseal. Several studies in liposomes have shown that a pore in a membrane can reseal spontaneously if the tension is low enough. It can persist if the tension equilibrates the hydrophobic forces that drive lipids to reassemble, or can grow and trigger the burst of the liposome if the tension is high [Taupin 1975, Zhelev 1993, Moroz 1997, Sandre 1999].

We saw in a previous section than cytoskeleton is necessary to maintaining membrane homeostasis. However, from a physical point of view, the interaction between the plasma membrane and the cortical cytoskeleton and the extracellular matrix increases considerably membrane tension [Raucher 1999, Dai 1999]. As seen previously, these interactions oppose to line tension and, therefore, also to membrane reseal, making membrane reseal a more challenging process. See Figure 3.6. It is likely that very small holes (< 0.2 μm) can still reseal spontaneously. Furthermore, it is also likely that in adherent cells where a more energy costing shape is imposed to the plasma membrane, resealing is more difficult that in non adherent round cells [12]. The cytoskeleton plays thus a dual role in the cell reaction to membrane wounding: unfavorable at very early stages where membrane physics is predominant (triggering self-reseal or expansion of the hole), favorable at later stages, when cellular machineries are addressed.

Altogether, cellular mechanisms are needed for most resealing processes to be

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12. Consistently, physical experiments have suggested that adhesion of vesicles to a substrate opposes to self-reseal and may lead to bursting of the vesicle if adhesion is too strong [Sandre 1999].

successful.

2.2.3 The cell understood physics: facilitating reseal by decreasing membrane tension

Tatsuru Togo and colleagues measured the membrane tension of 3T3 fibroblasts that underwent membrane wounding with laser or glass micro-needle. The tension was measured by sticking a bead to the membrane, trapping it with an optical tweezer, pulling a membrane tether, and estimating the force exerted by the membrane tether on the bead. They observed that plasma membrane tension of 3T3 fibroblasts decreased dramatically upon plasma membrane wounding. Interestingly, this drop in tension was Ca^{2+} dependent suggesting that Ca^{2+}-dependent responses to plasma membrane wounding are responsible for lowering membrane tension [Togo 2000].

Additionally, on the one hand, previous work showed that Ca^{2+}-dependent exocytosis is necessary for efficient plasma membrane repair in both invertebrate embryos and mammalian cells wounded by laser, glass micro-needle, electroporation or pore forming molecules [Steinhardt 1994, Bi 1995, Miyake 1995, Bi 1997, Togo 1999, Togo 2000, Huynh 2004, Tam 2010]. On the other hand, other studies showed that Ca^{2+}-dependent exocytosis lowers plasma membrane tension [Hagmann 1992, Dai 1997]. Altogether, these observations strongly suggest that a fundamental cellular response to plasma membrane wounding is the supply of membrane through Ca^{2+}-dependent exocytosis in order to lower membrane tension and facilitate reseal. Additionally, endocytosis and exocytosis may also be important in this process for the regulation of the membrane composition. Interestingly, rapid endocytosis and exocytosis are also involved during cytokinesis as reviewed by [Montagnac 2008, Echard 2008]. The previously described "caveolae membrane reservoir" may constitute another regulated local source of membrane to favor membrane repair. Consistently, recovery from wounding involves also a transient disassembly of the cortical actin locally at the wound site [Godin 2011, Miyake 2001, Togo 2000], perhaps involving proteins severing actin in a Ca^{2+}-dependent fashion such as calpain [Godell 1997]. Note that simple leaking-out of some amount of cytoplasm may also contribute to lower membrane tension as predicted by physical models in simple systems [Sandre 1999].

The clearance of cortical actin is thought to facilitate the approach of vesicles to patch wounds or to supply membrane in order to lower membrane tension. It is also likely that this local disruption of membrane-cytoskeleton interactions is a powerful mechanism by itself to promote the decrease in membrane tension and favor reseal.

2.2.4 Facilitated and potentiated resealing

T. Togo and co-workers have shown that when the plasma membrane is wounded twice at the same time, the second wound reseals faster. This facilitated reseal seems to be based on the generation of new vesicles after the first wound. These vesicles

are produced in a PKC [13]-dependent and in a Brefeldin A (BFA) sensitive manner [**?**], suggesting that these vesicles are produced by the Golgi apparatus. Consistently, lowering the temperature to 20°C (which blocks traffic) also suppressed the facilitation. Moreover, the activation of PKC by phorbol ester [14] improves the reseal of the first wound.

However, in the case where the second wound was performed elsewhere, the potentiation does not depend neither on the PKC pathway nor on BFA-sensitive pathways. This form of potentiation can be divided in short-term potentiation and long-term potentiation. Short term potentiation depends on cAMP and protein-kinase A (PKA) and long-term potentiation depends on the activity of CREB [15] [Togo 2003].

2.2.5 Resealing large holes with intracellular membrane patches and cytoskeleton rearrangements

Several studies in the membrane repair field have been done in eggs or oocytes from invertebrate animals. Most of these studies have used micropipettes to create large holes of about 10 μm in diameter, which are mainly sealed by membrane patches made of vesicles. In 1997, M. Terasaki and co-workers were the first to make the hypothesis of a sealing patch, using starfish oocytes and sea urchin eggs [Terasaki 1997]. This hypothesis was further confirmed by P. L. McNeil and co-workers [McNeil 2000, McNeil 2001]. It was first thought that lysosomes and derived organelles were the main responsible organelles for patch formation [Jaiswal 2002, Reddy 2001], but it was later discovered that almost any organelle could participate to this emergency response, as even proteins from the endoplasmic reticulum were found in the patches [Borgonovo 2002, Shen 2005, Bement 2007].

Moreover, apart from cortical actin clearance of the wound region to allow docking of vesicles and/or a decrease in membrane tension, the actin cytoskeleton is further reorganized during wound repair. In wounded early drosophila embryos or xenopus oocytes, an acto-myosin ring forms around the wound. This ring contracts until it closes into itself. The time the ring takes to contract is around 20 minutes, which is very long for a hole to remain open at the plasma membrane of an organism. This suggests that this mechanism is not a sealing mechanism but probably a way to get rid of a previously formed repair patch [Mandato 2003, Clark 2009, Abreu-Blanco 2011].

Recent studies have shown that MG53 is essential for vesicle trafficking during sarcolemma repair in muscle fibers or plasma membrane repair in C2C12 cells. MG53 localizes at the wound site, suggesting a patch-mediated repair [Cai 2009]. Nevertheless we cannot exclude the possibility that these vesicles act locally as membrane tension modulators instead of as patch forming vesicles.

Finally, observations made in oocytes and eggs of invertebrates have to be considered carefully when applying them to other types of cells because crucial differences exist

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13. PKC stands for protein-kinase-C. This family of proteins is activated by signals such as an increase of diacylglycerol or Ca^{2+} concentration

14. Phorbol esters are plant-derived organic compounds that mimic diacylglycerols and activate PKC.

15. CREB stands for cAMP response element-binding protein.

between the different systems: 1- the oocytes and fertilized eggs used here are much larger than regular mammalian cells, 2- their round shape is likely to be less energy costly, with less cortical cytoskeleton and lower membrane tension, facilitating reseal and allowing large holes for seconds without bursting of the cell, 3- the holes performed are about 20 times bigger (>1000 μm^2) than the holes performed in mammalian cells.

It is very likely that the mechanisms of repair are different depending on the size of the hole and the cellular model studied. Indeed, very small holes generated by tuning electroporation are likely to reseal without Ca^{2+} [Muller 2003, Djuzenova 1996, Shirakashi 2002]. For holes of about 1μm and above, Ca^{2+} is required for reseal but not necessary a patch made of vesicles.

2.2.6 Dealing with small holes or wounds made by pore forming molecules

Patches are not incompatible with removal of pore-forming toxins if we consider a model where the patch replaces the portion of the membrane where the toxin is, and this portion is released in the extracellular medium. Nevertheless, an increasing number of studies suggest that pore-forming toxins, as well as small wounds, are handled rather by other mechanisms.

Pioneer work in cells permeabilized with the complement suggested two possible mechanisms to get rid of stable wounds such as pore forming toxins: 1- internalization [Carney 1985, Morgan 1987]; 2- externalization through blebs. Both hypothesis are still being considered [16].

The first hypothesis of endocytotic removal of pore-forming toxin is supported by recent work in the laboratory of Norma W. Andrews. The work of V. Idone and co-workers showed that endocytosis is required for the recovery of plasma membrane injured by Streptolysin-O or mechanically, in a cholesterol and Ca^{2+} - dependent manner and was degraded *via* MVBs in an ESCRT-III dependent manner [Idone 2008b, Tam 2010, Corrotte 2012]. Consistent with this observation, this group also showed that exocytosis during plasma membrane wounding promotes endocytosis [Tam 2010]. This is mediated by the release of the enzyme acid sphingomyelinase (ASM) by exocytosis. The conversion of sphingomyelin into ceramide is likely to promote positive curvature and budding in membranes *in vitro* and *in vivo*. See [Gulbins 2003, van Blitterswijk 2003, Grassme 2007] for review. Ceramides are also important for the generation of a subpopulation of ILVs [Trajkovic 2008]. These positive

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16. The difference between a bleb and a bud has to be taken into consideration, as the bleb term has been used to describe both phenomenons. In our view, a bleb is a much larger structure than a bud. A bleb appears when the cortical cytoskeleton is fragilized (usually by mechanical factors) or downregulated through controlled cellular mechanisms like during mitosis or migration [Charras 2008, Fackler 2008, Tinevez 2009, Norman 2010]. Once detached from the cytoskeleton, the portion of the membrane corresponding to the future bleb will tend to adopt a low energy morphology, *i.e.* the closest structure to a sphere that the portion of membrane can achieve while still being attached to the rest of the plasma membrane. What we usually call bud is smaller structure. In the cell, most structures we denominate buds are dependent on scaffolding mechanisms such as caveolin, clathrin or ESCRTs.

and negative curvature generation are not incompatible. Consistently, in the case of budding at MVBs, conversion of sphingomyelin into ceramide is achieved by neutral sphingomyelinase 2 which is cytosolic, while during wound repair ASM acts extracellularly [17]. Similar endocytic mechanisms to remove pore-forming proteins have been described for perforin [Thiery 2010], and pore-forming toxins such as *Staphylococcus aureus*-toxin and *Vibrio cholera* cytolysin [Gutierrez 2007, Husmann 2009].

However, even if the Andrews group could not detect an involvement of blebs imaged by light microscopy in the removal of Streptolysin-O (SLO), recent studies have shown that pore-forming toxins can be removed by budding-mediated externalization, with buds considerably smaller. Indeed, SLO can be detected on the buds by scanning electron microscopy [Keyel 2011]. This externalization was already proposed [Babiychuk 2009] with observations by fluorescence microscopy. These studies suggest that budding may be a novel mechanism to get rid of pore-forming molecules. This mechanism seems to be more healthy to the cell if we consider a cell as a selfish unit and not as a part of a whole organism. Indeed, internalizing pore-forming toxins as it was proposed by [Idone 2008b, Corrotte 2012] seem more challenging for the cell as it will involve a permeabilized endosome intermediate prior to the lysis of the pore-forming protein. It may be the case of *Vibrio cholera* cytolysin, which triggers the formation of large cytoplasmic vacuoles in intestine epithelial cells and triggers sometimes cell death [Coelho 2000, Figueroa-Arredondo 2001, Gutierrez 2007]. Actually, some bacteria have even adapted they pore-forming toxins so they are rather active inside the cell. For example, the activity of listeriolysin O is pH-dependent and, therefore, it is active only once it is endocytosed [Geoffroy 1987]. Finally, other non-proteic pore-forming molecules are likely to resist to the cellular degradation mechanisms, perpetuating, therefore, the permeabilization of membrane from organelle to organelle in a never ending struggle.

2.2.7 Actors of membrane repair

As it was described in previous sections, actin plays a major role for the contractile-ring formation during patch mediated repair (together with myosin), and its depolymerization is essential for vesicles to approach the plasma membrane for exocytosis. Intermediate filaments play a role probably for membrane resistance to damage rather than for repair. Additionally, microtubules play a crucial role during plasma membrane repair: upon needle wounding of PTK cells, the entry of Ca^{2+} triggers depolymerization of microtubules which are further organized as a radial spindle emerging from the wound perimeter [Mandato 2003, Togo 2006]. This organization of microtubules will probably allow specific and fast targeting of vesicles to the wound site.

Once at the wound site, vesicle fusion with the plasma membrane is mediated by SNAREs. Indeed, inhibition of membrane repair by toxins targeting SNAREs [Steinhardt 1994] and by expressing dominant negative synaptotagmin constructs [Shen 2005]. This mechanism may be valid in other organisms like plants where the

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17. It can not be excluded that cytosolic sphingomyelinases react also to plasma membrane wounding triggering budding of the plasma membrane towards the extracellular medium.

synaptotagmin syst-1 is involved in repair [Schapire 2008].

Several Ca^{2+}-regulated proteins have been shown to be indispensable for membrane repair. Dysferlin, for instance, is a Ca^{2+} calcium binding protein that has been implicated in fusion events in *Caenorabditis elegans*. Its mutations have been associated with muscular dystrophy cases [McNally 2007] and with wound sealing in knock-out mice [Bansal 2003]. Annexins A1 and A2, which are Ca^{2+} regulated and lipid binding proteins, interact with Dysferlin [Lennon 2003]. Moreover, at least Annexin A1 is required for membrane repair in HeLa cells wounded with a laser[McNeil 2006]. Annexin A1 seems also important for the response to Streptolysin-O mediated disruption of the plasma membrane [Babiychuk 2009]. It is likely that annexins interact with SNAREs as Annexin A2 interacts with them *in vitro* in a Ca^{2+}-dependent manner [Wang 2007]. Other proteins like MG53 have been shown to be important to drive vesicles to the wound site [Cai 2009] but the connection to Annexins and to SNAREs has not been investigated as far as I know.

Article manuscript 1:
A novel role for ESCRT-III proteins in plasma membrane repair

Objectives of the work

This part of our work has for aim to further elucidate the cellular responses to small damage occuring at the plasma membrane. We used several means to generate small holes in the plasma membrane of HeLa cells. We observed that large holes are poorly repaired. Our data suggest that stricking convergences exist between cytokinesis and virus budding on the one hand, and the observations made during plasma membrane repair on the other hand. Some of them are such as: the rapid endocytosis and exocytosis events accompanying both cytokinesis and membrane repair, the budding of the plasma membrane containing the pore forming protein Streptolysine-O. We identified in this work one more striking similarity between cytokinesis, virus budding and plasma membrane repair. We show here that ESCRT proteins are involved in the removal of injured portions of plasma membrane.

The results of our study are presented in the following manuscript. This manuscript is in preparation and the format are of the figures and the text may change before submission.

CHMP3 depletion seems to have a negative effect on ESCRT-III disassembly
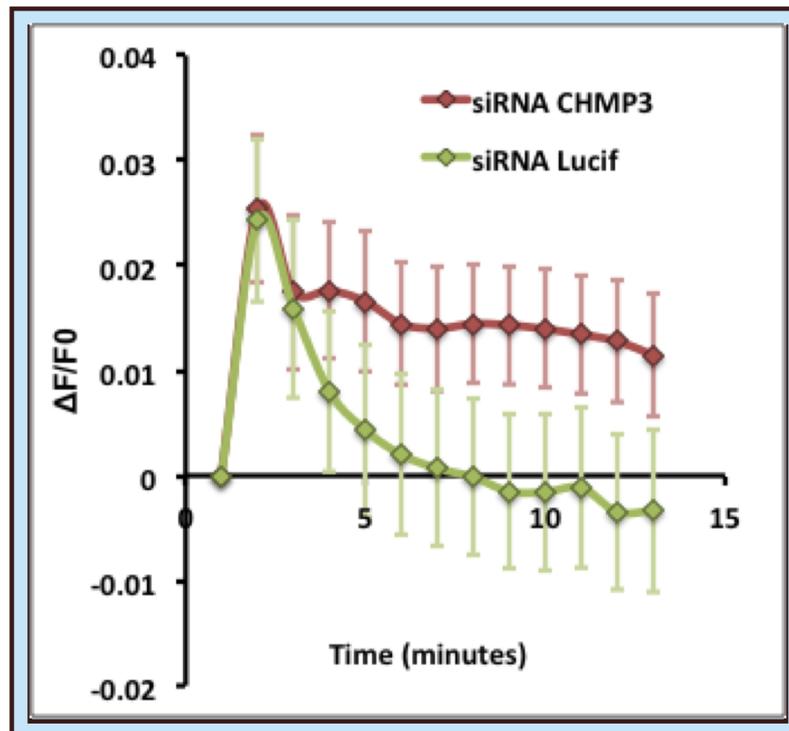
FIGURE 2.7 – **Recruitment of CHMP4B to laser wounds in cells treated with siRNA against CHMP3**

HeLa Kyoto CHMP4B-EGFP cells were wounded and imaged with a spinning disk microscope equipped with a UV laser module. CHMP4B recruitment at the wound was quantified in at least 40 cells. The resulting graph suggests that CHMP4B stays longer at the wounding site in cells treated with siRNA against CHMP3 than in cells treated with control siRNA. Although this result has to be confirmed, it is in agreement with the fact that CHMP3 possess a MIM1, which is shared with CHMP2 and which allows the to binding of CHMP2 to Vps4 (ESCRT-disassembly) *via* this motif. It is then possible that CHMP3 participates also to the recruitment of Vps4 by binding through its MIM1 motif.

In this experiment we used the following previously published siRNA sequence [Bache 2006]: 5'- AAA GCA UGG ACG AUC AGG AAG -3'.
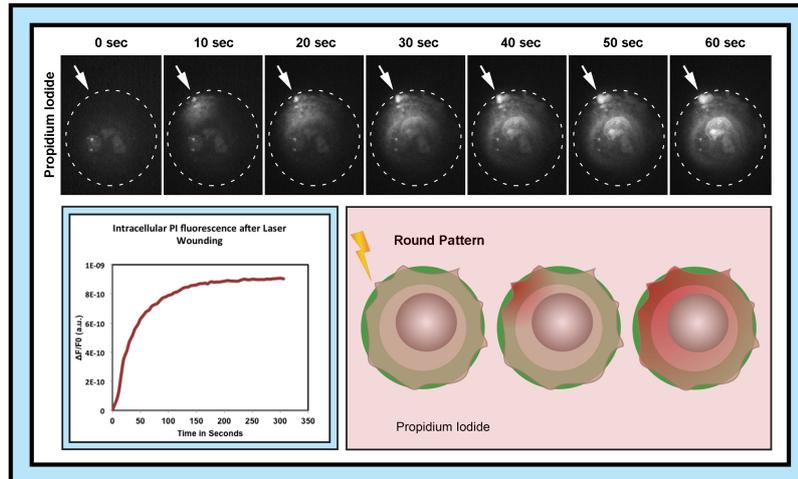
FIGURE 2.8 – **Semi-automatized analysis of PI entry upon wounding of the plasma membrane as a tool to study the dynamics of reseal in correlation with the size of the hole.**

Automatized PI-entry quantification for future modelization.

This figure show a 10 seconds - time-lapse imaging of the entry of Propidium Iodide (PI) upon laser wounding. HeLa Kyoto cells were platted on patterns and were wounded with a UV laser in the presence of PI at $160\mu g/ml$ to standardize as possible the measurement conditions. This assay is being automatized in terms of imaging with the patterns and analysis with the help of ImageJ macros, Visual Basic and MatLab. We aim to study the time of wound reseal (estimated by the time where PI reaches a plateau) and the diameter of hole (estimated qualitatively by the initial slope of the PI entry curve). This results are very preliminary and therefore will not be presented in detail here, but our preliminary quantification suggests that, at least in a certain range of wound size, the time of resealing is inversely correlated to the diameter of the hole. Although larger holes may be more difficult to reseal, their presence probably induces a more strong signaling and, therefore, a more efficient response from the cell. It is very likely that cells reseal small and large wounds with different machinery. It is also likely that the cell responds with different machineries to wounds with much smaller differences in size.
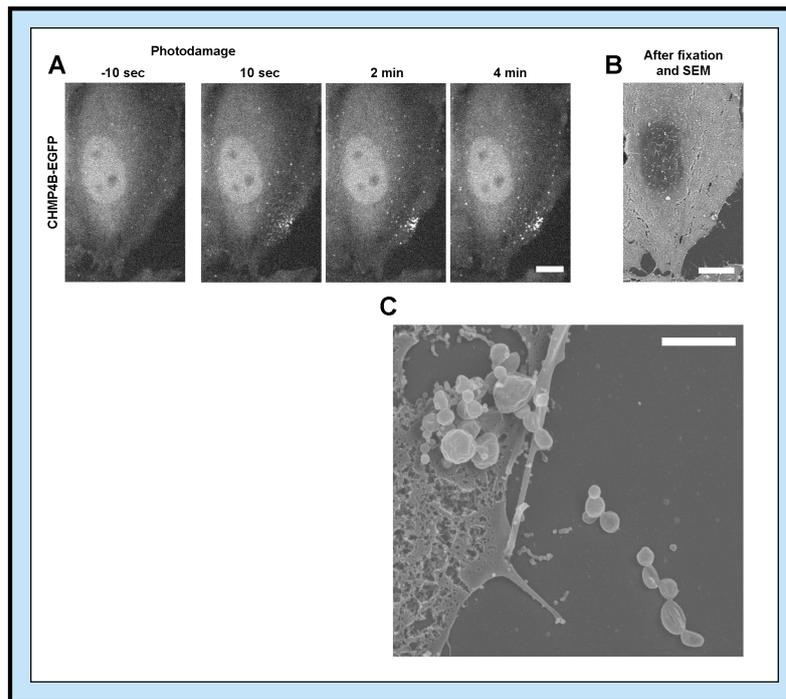
FIGURE 2.9 – **Shedding at ESCRT positive spots observed by fluorescence and correlative scanning-microscopy upon wounding of the plasma membrane supports the hypothesis of ESCRT-mediated shedding of damaged membrane posrtions.**

HeLa Kyoto CHMP4B-EGFP cells were platted on MatTek dishes labelled with grids. A- UV-laser mediated wounding of cells plasma membrane was followed by time-lapse imaging with a spinning disk microsocope to follow CHMP4B recruitment. Cells were subsequently fixed and processed for scanning electron-microscopy. Scale bar for A and B: 10 μm. Scale bar for C: 1 μm.

ESCRT-positive structures associated with membrane shedding observed by correlative scanning electron-microscopy.

As described previously in the paper manuscript, we performed correlative scanning microscopy on cells wounded by a laser beam and subsequently analyzed for CHMP4B recruitment. This assay revealed the presence of membrane buds a the wound site, which co-localize with ESCRT-positive protrusions. This strongly suggests that this buds are positive to and probably generated by ESCRTs. Shedding of ESCRT-positive particles was also observed. This figure shows tubular-budding membranes detached from the cell and locate close to the wound site, strongly supporting the hypothesis of ESCRT-mediated expulsion of injured membranes.

Part II

Dealing with damaged trafficking organelles: an example of the Golgi apparatus

Autophagy: A process for organelle elimination

Among the various threats that can damage the plasma membrane, and that I presented in chapter 3, many can also damage intracellular membranes, *i.e.* organelle membranes. We saw previously how the cell deals with plasma membrane wounds. It is obvious that the only way to deal with a wound in the plasma membrane is by repairing as elimination is not an option. In the case of organelles, a wound could be handled by two means: repair or elimination of the organelle. For the moment, no process of organelle membrane repair has been described. Moreover, besides potential wounds in the membrane, an organelle can also be impaired by other means such as the accumulation of proteins or an alteration of their biochemical composition leading, for example, to an aberrant pH in their lumen. Many drugs can affect organelle properties inhibiting essential processes like proton pumping. Thus, there are many ways to impair the homeostasis of an organelle that do not necessarily involve the injury of membranes. Dealing with organelle damage by elimination represents a generic response that can be applied to all kinds of damages. Elimination is possible because of the redundancy of most organelles. Autophagy has been shown to be involved in the elimination of some organelles like mitochondria and peroxisomes. Our work investigates its involvement in the handling of damaged trafficking organelles. In this chapter, I will present the different forms of autophagy and the mechanisms of autophagosome formation during macroautophagy and related selective mechanisms. The pathways controlling non-selective macroautophagy will not be presented here.

Contents

Figures

Tables

3.1   Autophagy: generalities

3.1.1   Definition

As its name indicates, autophagy is a process of eating (phagy) - itself (auto). It is a process where compounds of the cell itself are degraded inside the cell's lysosomes. It is an evolutionarily conserved mechanism among Eukaryots. By eating its own compounds, the cell has the ability to clean its cytoplasm from old long-lived proteins, aggregates, and old or damaged organelles. It is also able to recycle its compounds in situations of nutritional shortage. It has been further implicated in cell differentiation, remodeling and growth. See [Mizushima 2010] for review. It has also been involved in pathological situations such as aging, tumorigenesis, neurodegeneration and during viral or bacterial infections. See [Deretic 2009, Cuervo 2008, Levine 2007, Levine 2011, Levine 2008] for review.

Finally, if autophagy is mostly cytoprotective, extreme situations can lead the cell towards "autophagic cell death", also known as type II Programmed Cell Death (PCD) [Gozuacik 2007], another kind of cell death besides apoptosis and necrosis.

3.1.2   The different kinds of autophagy

Several mechanisms are included is the term autophagy. In mammals, we can define broadly four main sorts of autophagy: 1- chaperone-mediated autophagy, 2- microautophagy, 3- macroautophagy, 4- selective macroautophagy. This represents only generic categories because some of these categories include the elimination of specific cargo using specific receptors and signalling as we will see later on. Another kind of autophagy is the Cvt pathway which is a selective autophagy described in yeast. Nevertheless, this pathway has not been described in mammals and we will not extend its description in this work. Note that many stages of autophagy have been studied and understood in yeast where autophagic degradation does not occur in the lysosome but in the vacuole. Still, yeast and mammalian autophagy seem to share most mechanisms. See Figure 4.1 for visual comprehension of the different ways to deliver cargo into the lysosome by autophagy.
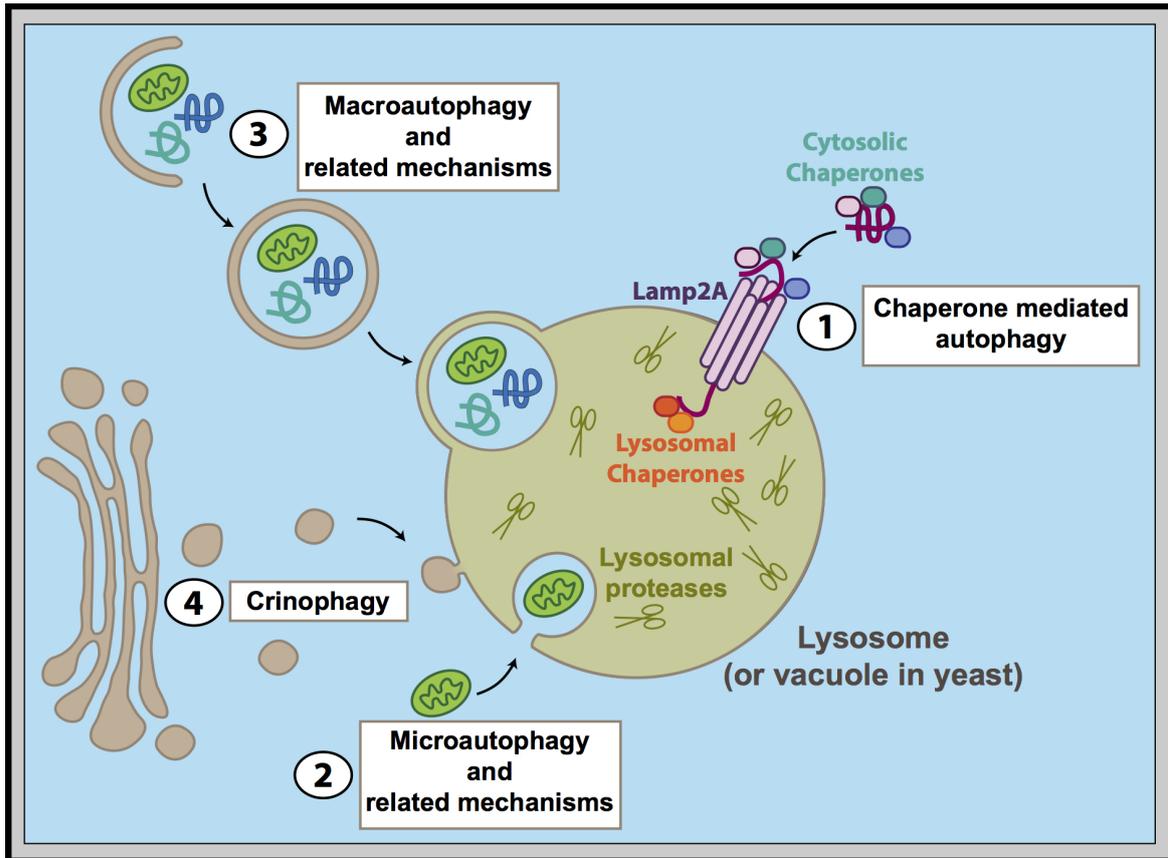
FIGURE 3.1 – **Different ways to deliver cargo into the lysosome.**
This figure represents the four ways that the different sorts of autophagy use to deliver cargo to degraden into the lysosome. 1- Chaperone mediated autophagy: direct delivery by chaperones of cytosolic proteins containing a KFERQ motif. 2- Microautophagy and related selective mechanisms: direct engulfment of soluble cytosolic compounds or organelles on the surface of lysosomes. 3- Macroautophagy: delivery of cargo transported in a double-membrane vesicle called autophagosome. 4- Crinophagy: direct fusion with the lysosome of Golgi derived secretory granules.

Chaperone mediated autophagy

The Chaperone-Mediated Autophagy (CMA) is responsible for selective degradation of certain of soluble proteins. Thus, this process is not involved in the elimination of organelles. CMA does not involve the surrounding of the cargo by any vesicle as target proteins are translocated directly into the lumen of the lysosome *via* a translocon. The proteins degraded by the CMA contain the KFERQ motif [Dice 1986, Dice 1990], which binds to the complex formed by the heat-shock chaperone [1] Hsc70 [2] [Chiang 1989] and other co-chaperones. The resulting complex associates then to the lysosomal protein LAMP2A on the surface of lysosomes [Cuervo 1996]. Target proteins are then translocated by chaperones and by the multimerized LAMP2A [Bandyopadhyay 2008]

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1. Heat-shock proteins are proteins activated by heat.
2. Hsc70 stands for Heat Shock Cognate 70kDa

into the lumen of the lysosomes where they will be degraded. See [Kaushik 2012] for review.

Microautophagy and related selective mechanisms

The microautophagy consists in the random engulfment of a portion of the cytoplasm directly at the surface of the lysosome. It was first described in rat liver by deDuve and Wattiaux in 1966. They described at the same time the phenomenon of macroautophagy [de Duve 1966]. The internalization of the soluble cytosolic compounds occurs through tubular invaginations [Muller 2000]. Equivalent selective processes also exist. See [Li 2012] for review. Each of them has been given a different name depending on the cargo that is selectively degraded. Some examples of microphagy-related selective mechanisms are:

1. micropexophagy, selective microautophagy for degradation of damaged or superfluous clusters of peroxysomes;
2. micromitophagy, selective microautophagy for mitochondria degradation [3];
3. piecemeal microautophagy of the nucleus (PMN), degrades non-essential portions of the nucleus.

The mechanisms for selective targeting differ in certain aspects. For example, the selective engulfment of organelles is rather mediated by arm-like structures than by invagination of the membrane of the lysosome. Additionally, the receptors ensuring cargo specificity are likely to differ from one process to the other.

Macroautophagy and related selective mechanisms

Macroautophagy is believed to be the main pathway to deliver cytosolic compounds into the lumen of the lysosome. It consists in the sequestration of cytosolic fractions by a double membrane vesicle called autophagosome. This autophagosome will fuse with lysosomes and/or late endosomes allowing the degradation of its content by lysosomal hydrolases.

This mechanism can be subdivided in the following steps (see Figure 4.2 for visual comprehension of these steps):

1. the induction of the process;
2. the formation of the first autophagosomal precursor membrane, the "phagophore";
3. the expansion of the phagophore into a pre-autophagosome, which starts surrounding the cargo to be degraded;
4. the closure of the pre-autophagosome into a double-membrane vesicle, the "autophagosome";
5. in some cases, the fusion of the autophagosome with late endosomes giving birth to an "amphisome";
6. the fusion of the autophagosome or the amphisome with lysosomes giving birth to the "autophagolysosome" where the cargo brought by the autophagosome will be degraded.
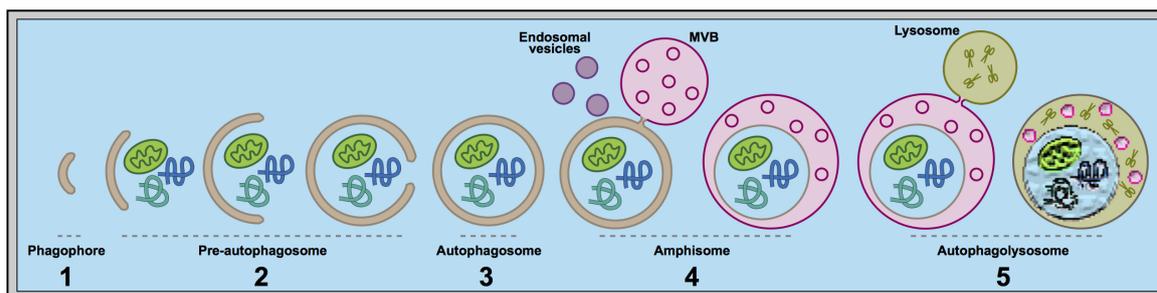
FIGURE 3.2 – **The autophagosome maturation**

This figure represents the life of an autophagosome from its biogenesis as a phagophore until its conversion into an autophagolysosome. 1- The first step is the formation of the cistern called phagophore, upon induction of the process. This structure if formed at the autophagosome formation site defined by a machinery of proteins. In the case of selective macroautophagy, it is possible that this phagophore is formed directly on the cargo to degrade. 2- The phagophore expands around the cargo and becomes a pre-autophagosome thanks to the addition of lipids and membrane of different organelle origin. This expansion is likely mediated by homotypic fusion aswell. 3- The pre-autophagosome closes into itself and gives a double-membrane vesicle called autophagosome. 4- The autophagosome may fuse with MVBs or endosomal vesicles to give an amphisome. 5- Finally the autophagosome or the amphisome fuses with a lysosome that brings the microenvironment and the proteases necessary for degradation.

Like microautophagy, this process has related selective mechanisms that degrade specific organelles (macropexophagy, macromitophagy, reticulophagy/ERphagy, zymophagy of secretory granules, lipophagy of lipid droplets), proteins (aggrephagy of protein aggregates, ribophagy, Vid pathway for selective turnover of fructose-1,6-bisphosphatase) or intracellular pathogens (xenophagy). See [Klionsky 2007] for review.

Crinophagy, a direct fusion of secretory granules with lysosomes

The Crinophagy is a type of autophagy that does not appear to follow any of the modes described above for the delivery of cargos into the lysosome. Crinophagy was first described as a process of direct fusion of secretory granules with lysosomes, giving a crinosome [Glaumann 1989]. Later on, it was discovered that this process is involved in the degradation of insulin stored in a vesicle [Schnell Landstrom 1991]. However, it is not clear whether the delivery of insulin into lysosomes is mediated by direct fusion between the secretory granules and the lysosome or if the insulin-containing granules are sequestered by an autophagosome before delivery to the lysosomes [Klionsky 2007]. Additionally, portions of the Trans-Golgi network containing oxidized apoB-aggregates are targeted and degraded by the autophagic machinery [Pan 2008], suggesting that trafficking organelles are prone to autophagic degradation.

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3. This phenomenon has been less studied than the degradation of mitochondria *via* autophagosomes.

3.2 The mechanisms of macroautophagy

Our study is focused on the early stages of selective autophagy. Therefore, the mechanisms of the degradation that take place inside the lysosome will not be presented here.

3.2.1 The autophagosome
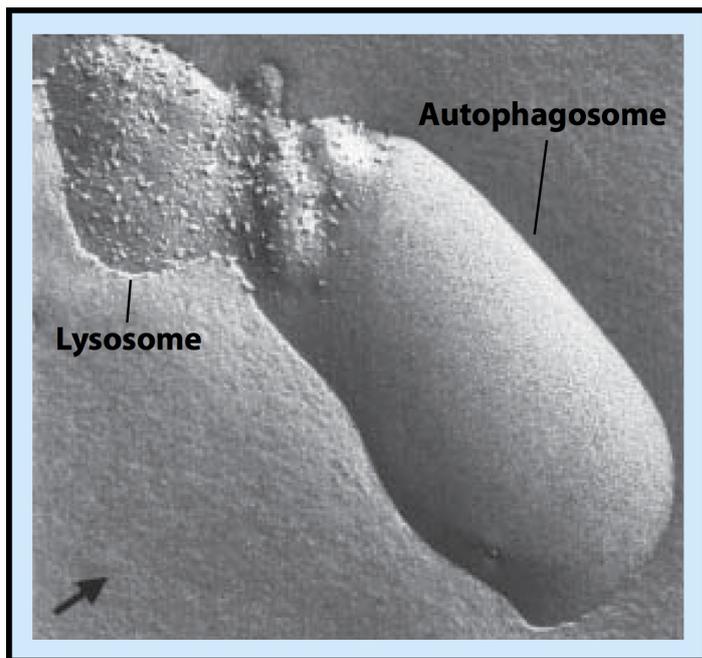
FIGURE 3.3 – **An autophagosome fusing with a lysosome.**

This image shows the fusion between an autophagosome and a lysosome observed by electron-microscopy after cryo-fracture. The texture of the surface of this two organelles is very different probably due to the presence of very few proteins at the smooth surface of the autophagosome. Adapted from [Fengsrud 2000].

The autophagosome is a double or multimembrane vesicle. It is usually about 700 nm in diameter, but it can become larger if the cargo to sequester is bigger, like organelles or bacteria [Xie 2007]. One characteristic of autophagosomes, which is actually shared with phagosomes, is the very small number of markers from other cellular compartments that can be found associated with autophagosomes [Mari 2011]. This has represented a big challenge for the understanding of the origin of these organelles. It is believed that the autophagosome contains remarkably few proteins on its surface as shown by electron-microscopy after cryo-fracture [Fengsrud 2000]. Actually, until now, only the protein LC3 (or Atg8 in yeast) has been described as a marker of fully closed autophagosome.

3.2.2 The origin of autophagosomes

Unlike all the rest of degradation processes where the organelles responsible for degradation already exist in the cell, during macroautophagy, autophagosomes are formed *de novo* upon induction of the autophagic process. The biogenesis of this organelle begins with the formation of the phagophore (or isolation membrane) which is a cistern-shaped membranous structure [Mizushima 2001, Militello 2011, Eskelinen 2011]. The Atg

("autophagy-related") proteins were first identified by genetic screens in *Saccharomyces cerevisae* as proteins necessary for the biogenesis of autophagosomes. It is believed that the phagophore is generated following the sequential association of some Atg proteins. This structure is called the "phagophore assembly site" or "pre-autophagosomal structure" [Xie 2007, Suzuki 2007, Yoshimori 2008, Itakura 2010, Mari 2010]. The origin of the membranes of autophagosomes is still controversial as several sources of membrane have been proposed.

The **endoplasmic reticulum (ER)** was the first candidate because transmembrane proteins from the ER were detected on autophagosomal membranes [Dunn 1990]. This model is supported by the observation of connections between the endoplasmic reticulum and growing phagophores [Hayashi-Nishino 2009, Yla-Anttila 2009, Fujita 2008a] positive for Ulk1(Atg1 in yeast), Atg14L, Atg16L, LC3(Atg8 in yeast), and WIPI1 and WIPI2 (two mammalian homologues of yeast Atg18) [Itakura 2010, Axe 2008, Polson 2010]. These connections are spatially linked to PdtIns-3-P enriched subdomains. Moreover, Atg14L, a subunit of the phosphatidylinositol-3-kinase (PI3K) complex is localized at the ER membranes [Matsunaga 2010]. Its requirement during macroautophagy has been identified by the use of blocking drugs such as 3-methyl-adenine or Wortmannin [Petiot 2000, Seglen 1982]. Interestingly, the protein p62 (*a.k.a.* SQSTM1), which is mainly considered as an adaptor protein between Ubiquitin and mammalian Atg8 family, localizes to the autophagosome formation site at the ER during starvation induced macroautophagy [Itakura 2011]. It has been suggested that apart from its adaptor role described previously,, p62 may contribute to the nucleation of autophagosomes during starvation. p62 may also be able to nucleate autophagosome formation on protein aggregates through its interaction with ALFY [4] [Clausen 2010]. In turn, ALFY interacts directly with Atg5 and PtdIns3P and probably recruits that way the autophagosome formation machinery [Filimonenko 2010]. Therefore, probably by recognizing some cargo to be degraded, p62 may be an important factor for the phagophore biogenesis.

The **mitochondria** is also a candidate for the supply of membrane for autophagosomes, at least in aminoacid starvation conditions. This model was recently proposed by D. W. Hailey and co-workers. It is based on extensive-fluorescence microscopy analysis, where autophagosomes grew very close to mitochondria and lipids from mitochondria seemed to be transferred to the growing autophagosomes [Hailey 2010]. Nevertheless, it remains unclear whether mitochondria constitutes or not a spot for phagophore biogenesis, besides a lipid supplier probably contributing only to autophagosome expansion [Mari 2011].

The **plasma membrane** was proposed to be necessary for the formation of autophagosomes [Moreau 2011]. This work showed that the interaction between Atg16L1 (an Atg protein required for autophagosome biogenesis) and Clathrin heavy

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4. ALFY stands for autophagy-linked FYVE

chain is essential to reach early stages of the autophagosome biogenesis. SNAREs found at the plasma membrane appear also to be necessary for this biogenesis [Moreau 2011, Nair 2011], as well as the exocyst complex[5]. Although, this may suggest a link with plasma membrane dynamics, Clathrin, SNAREs and exocysts have been observed in other compartments than the plasma membrane.

Finally, the **Golgi apparatus** and related compartments (trans-Golgi network and endosomes) are also candidates because proteins essential for starvation induced autophagy such as Atg9 (an essential protein for the phagophore assembly site in yeast [Young 2006, Mari 2010, Ohashi 2010]) and the exocyst complex localize at the trans-Golgi network, post-Golgi vesicles and endosomes [Bodemann 2011, Geng 2010]. Other arguments are: 1- the presence of lectins at the surface of autophagosomes that recognize glycans that are otherwise only found in membranes of the post-Golgi network [Yamamoto 1990]; 2- the fact that some Golgi associated proteins (lobe B of the COG complex[6] and some subunits of the TRAPIII complex[7] are essential for autophagy [Lynch-Day 2010, Yen 2010, Mari 2011]. 3- the Golgi protein GATE-16[8] (*a.k.a.* GABARAPL2), is important for autophagosome biogenesis [Weidberg 2010] and interacts with Golgi proteins like the v-SNARE GOS-28 [Sagiv 2000]. 4- finally, very recently the drosophila Golgi protein Ema has been shown to be essential for mitophagy [Kim 2012].

3.2.3 The phagophore expansion and completion

The sources of membrane

Once the Phagophore is formed, this cistern-like membrane has to grow to be able to surround the cargo to be degraded. It is not clear how this expansion takes place, but there are two models that are likely to coexist. The first model consists in the direct flow of lipids from other organelles such as the ER or the mitochondria. As we saw before, there exist connections between the ER and the growing autophagsomes [Hayashi-Nishino 2009, Yla-Anttila 2009, Fujita 2008a] and lipid flow has been observed from mitochondria towards autophagosomes [Hailey 2010]. The second model relies on fusion between phagophores, pre-autophagosomes, and/or fully formed autophagosomes, and is therefore compatible with the requirement of SNAREs for autophagosome biogenesis [Moreau 2011, Nair 2011]. Nevertheless other mechanisms for fusion may be involved in this process. The class III PhosphoInositide 3-kinase (PI3K-III), which converts PhosphoInositide(PI) into PhosphatidylInositol-3-Phosphate (PtdIns3P or

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5. The exocyst is a tethering complex that mediates the fusion of secretory vesicles from the Golgi apparatus with the plasma membrane. It acts together with the SNAREs proteins.

6. COG stands for Conserved Oligomeric Golgi complex, which is thought to play a role in intra-Golgi retrograde trafficking, which is ensured by COPI (Coat Protein I vesicles).

7. TRAP stands for TRAnsport Protein Particle. The TRAPP complexes are guanine-nucleotide exchange factors for RAB1 (or Ypt1 in yeast). TRAPPI is required to tether coated vesicles during ER to Golgi transport. TRAPPII has the same role during intra-Golgi traffic. TRAPPIII has been resently involved in autophagy (see above).

8. GATE-16 stands for Golgi-associated ATPase enhancer of 16kDa.

PI3P) is essential for the formation of autophagosomes [Petiot 2000, Kihara 2001]. Its inhibition by drugs like 3-Methyl-adenine or Wortmannin inhibit autophagosome formation [Petiot 2000, Seglen 1982].

The protein LC3 (Atg8 in yeast)

The yeast Atg8 protein is crucial for autophagosome expansion. Some studies suggest that it plays a role in this elongation by mediating hemifusion between autophagosomes and/or autophagosome precursors [Abeliovich 2000, Nakatogawa 2007, Xie 2008]. Atg8 has several orthologs in mammals that can be divided in two subgroups using the homology in their amino acid sequences [Xin 2001, He 2003]: 1- the first subgroup includes LC3A (which has two splicing variants), LC3B and LC3C; 2- the second subgroup includes GABARAP[9], GABARAPL1, GABARAPL2 (also known as GATE-16).

The GABARAP subgroup is involved in autophagosome maturation while the LC3 subgroup is rather involved in autophagosome elongation. It is very likely that LC3A, B and C ensure tissue specialization as they present different patterns of expression [Wu 2006]. Among the LC3 subfamily, the protein LC3B has been the most studied, but all LC3 proteins appear to follow the same processing and behavior.

In yeast, the C-terminal arginine residue of Atg8 is cleaved by the cystein protease Atg4 [Kirisako 2000]. The exposed Glycine is then conjugated to a Phosphatidyl-ethanolamine (PE) [Ichimura 2000]. Atg8 can be deconjugated from the PE by the same cystein protease Atg4. This deconjugation is necessary for the maturation of autophagosomes [Kirisako 2000]. Like the yeast Atg8, all the mammalian homologues present two forms I (soluble) and II (membrane-bound). These steps are required for the localization on autophagosomes [Kabeya 2000, Tanida 2003, Kabeya 2004, Wu 2006]. We will focus, from now on, on the LC3 family, and we will call it LC3 for simplicity.

LC3 is present in three forms in cells:

1. pro-LC3, the precursor form of the protein, undergoes cleavage of its C-terminal 22-amino acid fragment soon after its synthesis [Kabeya 2000];
2. LC3-I, is a cytosolic protein and is the result of the C-terminus cleavage of the pro-LC3 by Atg4B [Kabeya 2004];
3. LC3-II, is a membrane bound protein and is the result of the conjugation of LC3-I with a PE, and is also a substrate for Atg4B [Kabeya 2004].

Interestingly the protein LC3 can, in some cases associate directly with the membranes of damaged organelles. It is the case in MEFs Atg9L1^{-/-} deficient, where LC3 is directly associated with the membrane of endosomes containing Salmonella [Kageyama 2011].The mutant LC3[G120A] cannot undergo cleavage. No LC3-I (and therefore no LC3-II) is produced in this case suggesting that the Glycine120 residue is necessary for the cleavage of the pro-LC3 [Kabeya 2000]. Note that Atg4B can efficiently cleave all LC3, GATE-16 and GABARAP whereas Atg4A cleaves efficiently GATE-16 [Scherz-Shouval 2003], but less efficiently GABARAP and even less efficiently LC3 [Kabeya 2004]. Interestingly, the expression of the dominant-negative

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9. GABARAP stands for gamma-aminobutyric-acid-typeA (GABA$_A$)-receptor-associated protein.

mutant Atg4B ^{C74A} impairs the lipidation of LC3 and its paralogues suggesting that it has a dominant-negative effect on its paralogue Atg4A [Fujita 2008a]. This construct is particularly useful to impair LC3 function while avoiding partial rescue by a possible redundancy between LC3 and its mammalian paralogues.

The Ubiquitin-like conjugation systems

Two Ubiquitin-like conjugation systems are involved in this expansion/completion of autophagosomes. They are required for LC3 lipidation (conjugation to PE). These two systems are presented in Figure 1.3 under the Ubiquitin conjugation system.

In the first Ubiquitin-like system, the protein Atg12 behaves as a Ubiquitin. It is attached to the E1-like protein Atg7, and is then transferred on the E2-like enzyme Atg10, and further transferred on the substrate Atg5. The complex Atg12-Atg5 will further associate with Atg16L to form the Atg16L complex. This complex will behave as an E3-like enzyme in the second Ubiquitin-like system. See [Shaid 2012] for review.

In this second system, LC3 (Atg8 in yeast) behaves as a Ubiquitin. It is first attached to Atg7 (E1-like enzyme), transferred to Atg3 (E2-like enzyme) and then transferred on a PE (the substrate). This last step is mediated by the Atg5-Atg12-Atg16L complex which, as we saw before, behaves as an E3-like enzyme. Recent studies have shown that Atg16L associates with the isolation membrane [Mizushima 2003]. It is therefore possible that the Atg16L complex brings the Atg3-LC3 intermediate to the PE substrate. It is also likely that the localization of the Atg16L complex on some phagophore subdomains determines the place where the expansion of the phagophore begins [Hanada 2007, Mizushima 2003, Fujita 2008b].

3.3 The mechanisms of selectivity in macroautophagy-related selective mechanisms

The increasing number of selective macroautophagy-related selective mechanisms challenges the existence of "non-selective" mechanisms. Selective mechanisms seem to rely on the same machinery for autophagosome formation. Besides, each selective-mechanism described appear to have its own particularities, especially for the selection of cargo. However, most if not all of them share one common feature: the involvement of ubiquitination and of Ubiquitin binding proteins.

3.3.1 Protein adaptors or autophagy receptors

The autophagy receptors are proteins able to make the link between cargo and autophagic machinery. These receptors usually also interact with the Atg8 family. The adaptor proteins usually contain a LIR-motif (LIR stands for LC3-Interacting Region). See [Johansen 2011] for review. It mediates the binding to all the proteins
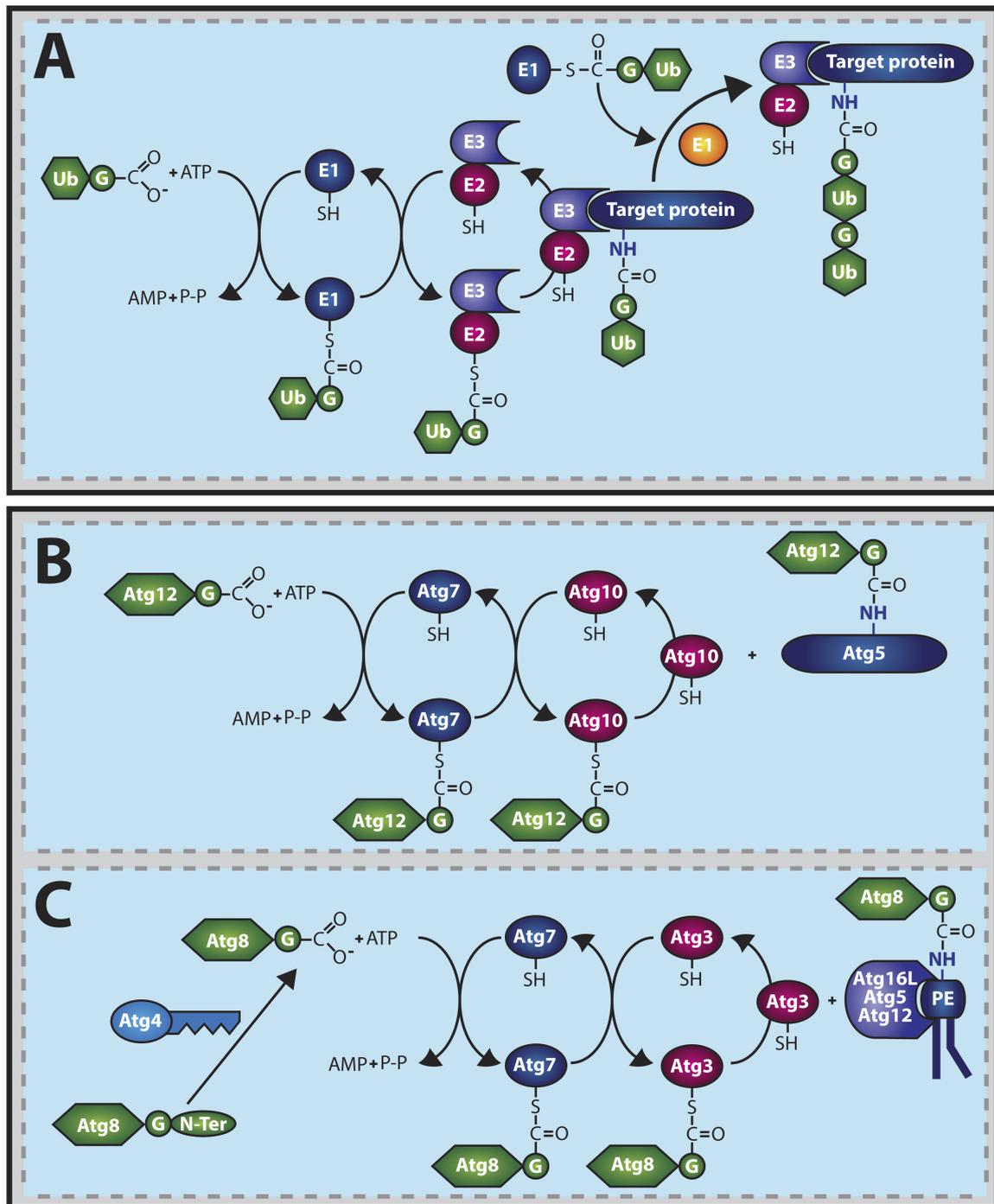
FIGURE 3.4 – **Ubiquitin-related mechanisms in autophagy**
This figure makes the parallel between the ubiquitination mechanism and the two ubiquitination-like mechanisms involved in autophagy.

of the mammalian Atg8 family. See [Johansen 2011] for review. In mammals, the most characterized autophagy receptors are p62 (*a.k.a.* sequestosome1 or SQSTM1)
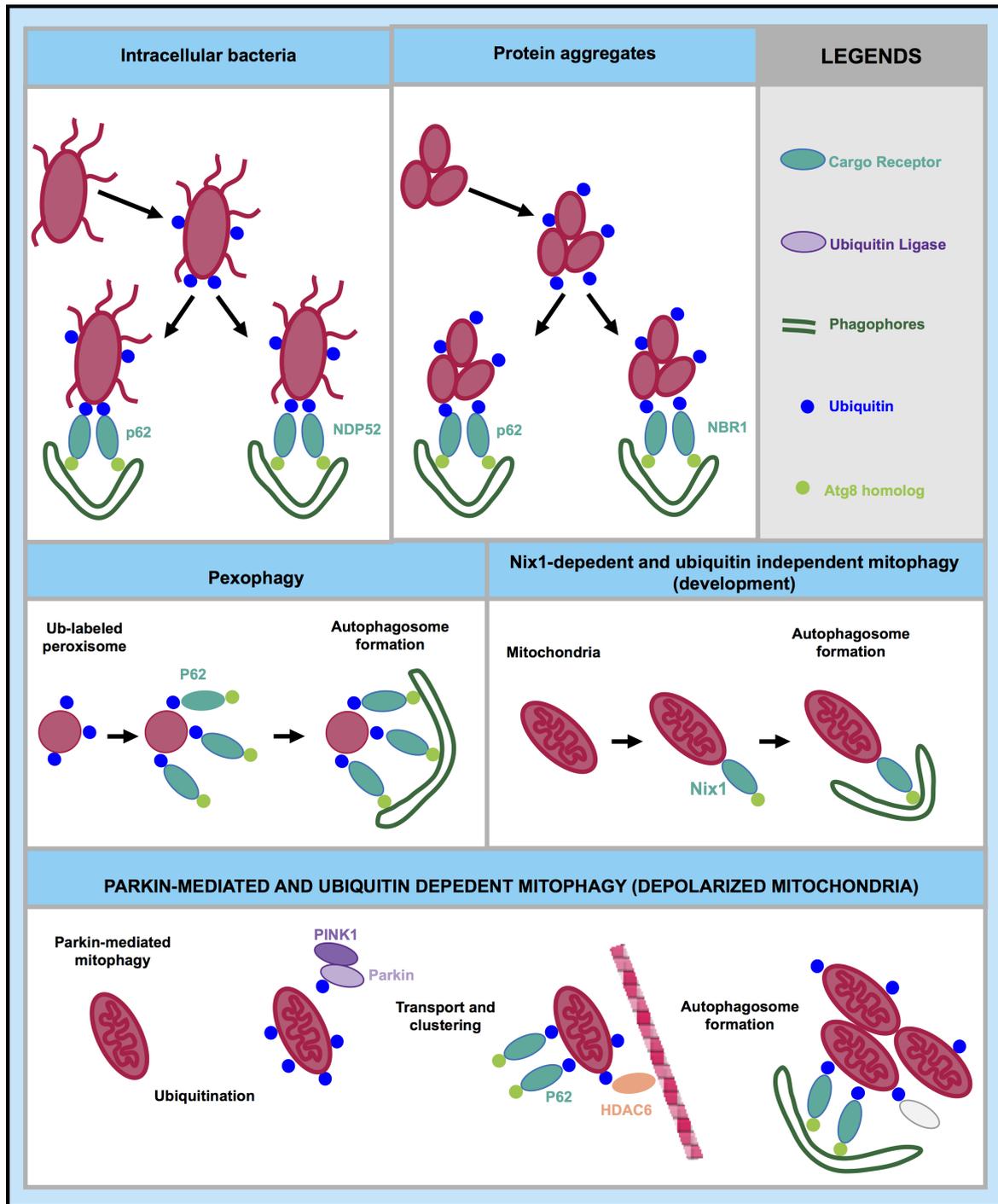
FIGURE 3.5 – **Some examples of autophagy receptors and substrates**
This figure shows some examples of autophagy receptors and their involvement in the elimination of different sort of cargos. Based on [Johansen 2011].

and NBR1 (neighbour of BRCA1) which both contain a UBA domain [10] and a LIR domain making the link between ubiquitinated cargo and Atg8-family proteins

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10. UBA domain stands for Ubiquitin-associated domain

[Bjorkoy 2005, Pankiv 2007, Kirkin 2009b, Kirkin 2009c, Kirkin 2009a]. These two proteins contain an oligomerization domain, PB1, [11] which allows the formation of ho-modimers, or heterodimers of proteins both containing a PB1 domain [Johansen 2011]. p62 and NBR1 complement each other for the selective elimination of protein aggregates during aggrephagy [Kirkin 2009b, Kirkin 2009a]. p62 has also been implicated in the degradation of several ubiquitinated substrates including damaged mitochondria [Ding 2010, Geisler 2010], peroxisomes [Kim 2008], intracellular bacteria [Zheng 2009], proteins from the viral capsid [Orvedahl 2010] and midbody remnants [Pohl 2009]. As seen earlier in this chapter, p62 may also play a role in the nucleation for phagophore formation, even in non-selective forms of macroautophagy [Itakura 2011, Clausen 2010, Filimonenko 2010]. Nevertheless, p62 does not appear to be crucial in these processes or at least during mitophagy which still occurs in p62 knock-out MEFs [Okatsu 2010, Narendra 2010].

Other LIR-containing autophagy receptors include NDP52 which is necessary together with p62 to restrict intracellular replication of *Salmonella typhimurium* [Zheng 2009, Thurston 2009]. These two proteins are recruited to different subdomains of the bacteria [Cemma 2011] and are also involved in the elimination of Listeria and Shigella [Mostowy 2011]. Unlike p62 and NBR1, NDP52 does not have a Ubiquitin-binding domain, but can bind proteins that directly bind to Ubiquitin [Thurston 2009]. The Nix protein may play a similar role during macromitophagy. See [Johansen 2011] for review.

Moreover, other proteins involved in the autophagic machinery like Atg4B and Atg3 also contain LIR motifs. In the case of these two proteins, the LIR-mediated interaction with LC3 may be important to fulfill their role in cleaving and lipidating LC3 respectively [Satoo 2009, Yamaguchi 2010]. The LIR motif may aswell mediate degradation of some proteins like Dishevelled [Gao 2010].

3.3.2 Regulation of selective autophagy by Ubiquitin

The ubiquitination of several substrates such as protein aggregates, depolarized mitochondria or bacteria functions as a signal used to recognize the cargo to be degraded by the adaptor proteins described above. See [Johansen 2011] for review. Tagging of proteins at the surface of peroxisomes is also sufficient to target them for degradation in a p62 dependent manner [Kim 2008].

Interestingly, p62 binds preferentially mono-Ubiquitin rather than poly-Ubiquitin *in vitro* [Long 2008]. This is consistent with the higher affinity of p62 for Lys63-linked chains, which present a more open conformation than Lys48-linked chains [Seibenhener 2004]. In agreement with this, depolarized mitochondria are labelled with Lys63-linked poly-Ubiquitin chains [Geisler 2010, Okatsu 2010]. The proteins prone to ubiquitination and the responsible E3-Ubiquitin ligases are unknown in most cases. The E3-Ubiquitin ligases Parkin and RNF185 [12] have been shown to be implicated in macromitophagy [Narendra 2008, Tang 2011], and SMURF1 [13] has been

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11. PB1 domain stands for Phox and Bem1p domain
12. RNF185 stands for RiNg Finger protein
13. SMURF1 stands for SMAD ubiquitination regulatory factor 1 or SMAD specific E3-Ubiquitin

implicated in both macromitophagy and selective autophagic targeting on viral particles [Orvedahl 2011].

Other pathways like phosphorylation of LC3 are likely to regulate selective autophagy, by modulating the interaction of LC3 with adaptor proteins [Shvets 2008, Cherra 2010, Jiang 2010, Reggiori 2012]. Besides, another kind of regulation may be specific to the cargo to degrade. For example, it has been shown that elimination of depolarized mitochondria depends on oxidation and is inhibited in the presence of antioxidants like N-acetyl-L-cysteine [Ding 2010], which, to my knowledge, have only been reported during mitophagy.

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protein ligase 1

Article manuscript 2:
Autophagic response to the damage of trafficking organelles, the example of the Golgi apparatus

Objectives of the work

Most studies focused on organelle elimination by autophagy concern aberrant or superfluous peroxisomes, or depolarized mitochondria. Apart from few examples, very little is known about the selective elimination of damaged organelles. By damaged, I mean damaged by factors exogenous to the organelle and not difunctional. Only few studies of light-damaged mitochondria have been performed where LC3 was selectively targeted to the wounded mitochondria [Elmore 2001, Kim 2007b]. Given the archebacterial origin of this organelle, and its implication in energy production and in cellular death by apoptosis, it is likely that the cell reaction to mitochondrial damage differs from its reaction to the damage of other organelles like trafficking organelles.

Our study aims at further determining whether other damaged organelles like trafficking organelles can also be a target for autophagic elimination and how similar are the mechanisms involved compared to the ones already described for protein aggregates or mitochondria.

The results of our study are presented in the following manuscript.

Annexes 2

NBR1 is recruited to the damaged Golgi apparatus.
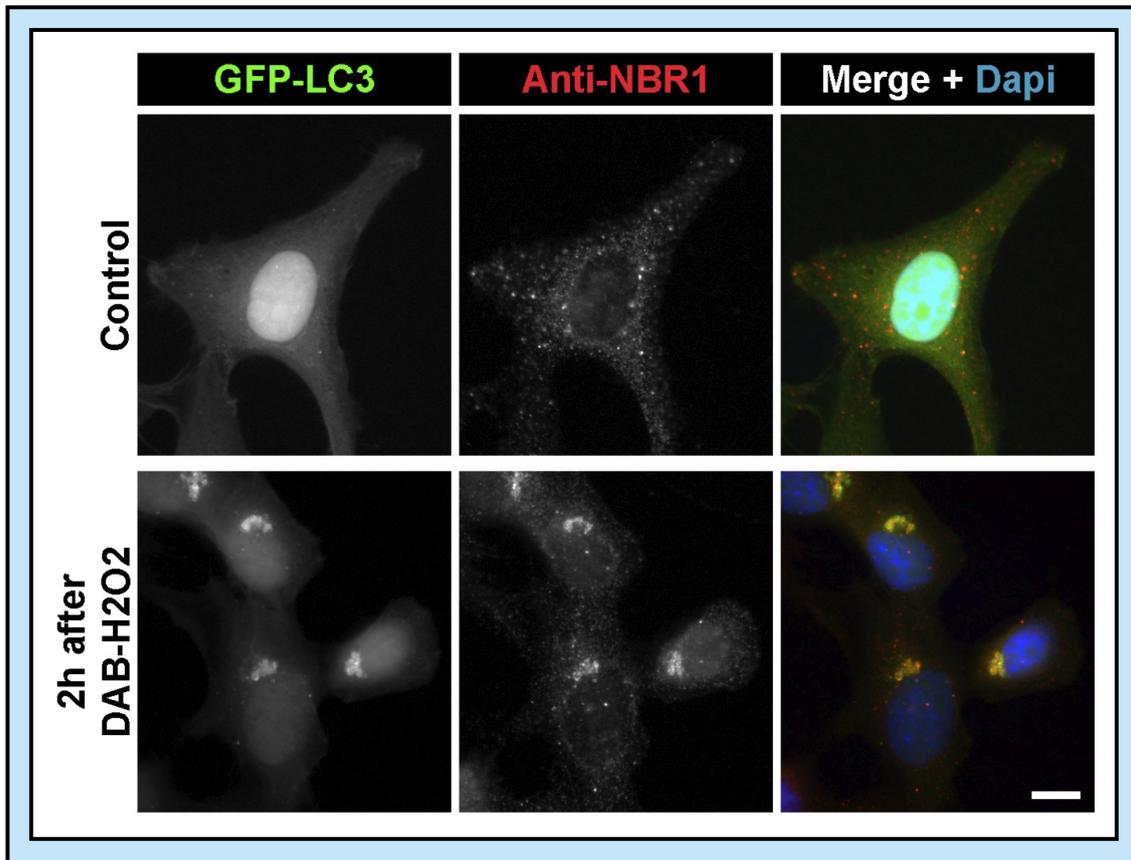
FIGURE 3.6 – **Recruitment of NBR1 at the cross-linked Golgi.**

NBR1 is a protein adaptor like p62, capable to bind ubiquitin and LC3. It possess a PB1 domain that allows its oligomerization [Kirkin 2009b, Kirkin 2009a]. It localizes to protein-aggregates positive for p62 [Kirkin 2009a, Kirkin 2009b]. However, very little is known about this protein and its role in autophagy. Our data shows that NBR1 is also recruited at the damaged Golgi apparatus. As NBR1 shares several characteristics with p62, we could think these two proteins may play a partial rundant role. This could explain why we still see LC3 recruitment in knock-out MEFs for p62. We performed siRNA against NBR1 in order to test this hypothesis, but all siRNA against NBR1 resulted very toxic. Interestingly, cells treated with siRNA against NBR1 were very sensitive to DAB-H_2O_2 based Golgi damage, and most of them died from necrosis soon after Golgi damage. This could be explained by two different hypothesis. First, it is possible that NBR1 is indeed an important molecule for cellular response to the damage of the Golgi apparatus. Second, it is possible that the cells were already very sensitive to any alteration. Nevertheless, both hypothesis suggest that NBR1 is a crucial protein for cell homeostasis.

Part III

General discussion

Repair or destroy? Keep or reject?

This thesis examines two paradigms of damage-induced cellular membrane repair: the plasma membrane and the Golgi membrane. The first one surrounds the cell while the second delineates an intracellular organelle. Diseases ranging from myopathies to neurodegeneration are associated with defaults in membrane repair mechanisms and selective autophagy and it is therefore evident that both mechanisms are crucial for cell survival and organism homeostasis. It appears that the cell has adapted very different mechanisms in response to similar types of damage to different membranes, that is the Golgi and the plasma membranes. The following points will be discussed in this chapter:

1. Is the observed involvement of the ESCRT in plasma membrane repair compatible with other roles previously described for ESCRTs?
2. How do our results reconcile with previous observations and models in the field of plasma membrane repair?
3. If damaged plasma membrane cannot be "eliminated", why are damaged intracellular membranes eliminated rather than repaired? What underlies the difference between the cell´ s responses to the damaged cell plasma membrane versus the damaged Golgi membrane?
4. Why is it important to eliminate handle damaged trafficking organelles?
5. How can we reconcile the differences observed between the handling of damaged Golgi apparatuses with previous models for the management of damaged organelles?
6. How do both mechanisms articulate to ensure cellular homeostasis? When should the cell keep damaged compounds, and when should it release them into the extracellular environment?

4.1 The role of ESCRTs in plasma membrane repair

In this study, we present evidence for the involvement of ESCRT proteins in plasma membrane repair. This is the 5th function attributed to the ESCRT, after ILV biogenesis, virus budding, cytokinesis and autophagosome closure (although the role of the ESCRT in autophagosome closure has not yet been verified). See Figure 5.1.

4.1.1 ESCRT subunit requirement and selective recruitment

Our results show a selective response of ESCRT proteins to plasma membrane damage. The increased mortality of cells depleted for ESCRT-III machinery reveals a
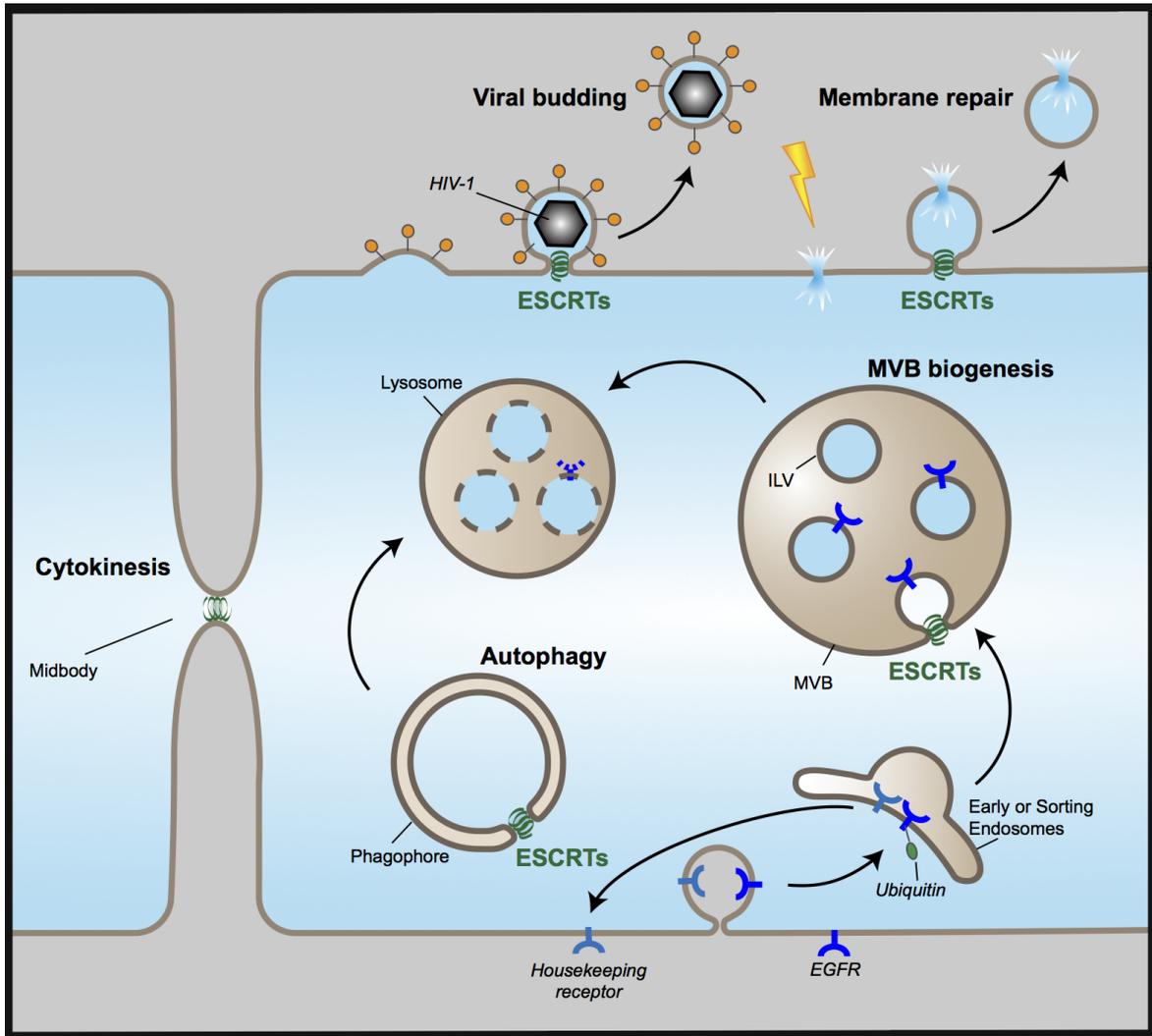
FIGURE 4.1 – **ESCRT proteins in cellular functions**

The present scheme presents all the functions attributed to ESCRTs including the one proposed by this work during plasma membrane repair. ILV biogenesis, virus budding, cytokinesis, autophagy (still a hypothetic role) and plasma membrane repair. Please note that the represented ESCRT helixes are well positionned and may not always accurately represent the reality. In the representation of ESCRTs during plasma membrane repair, it is not clear if ESCRTs act once the wound is repaired or if they eliminate an "open wound". The models for ESCRT action and how they reconciliate with previous models will be presented later on in this chapter and in Figure 5.2. Based on [Hurley 2010].

role for the ESCRT in the resistance of cells to plasma membrane damage. This partial increase in mortality is compatible with previous studies showing that only a subset of membranes wounded by pore-forming toxins are repaired by membrane shedding [Walev 1995]. Due to the implication of exocytosis which has been described in many models of plasma membrane repair, an initial hypothesis may be that ESCRTs are recruited to the plasma membrane wound as endosomal binding proteins; however, video microscopy experiments presented in this thesis suggest a recruitment of ESCRTs

from a cytosolic pool rather than a recruitment of ESCRT positive vesicles. This is supported by the fact that markers of lysosomes and late endosomes such as Lamp1 and Lysotracker do not collocalize with ESCRTs at the site of the wound. The experiments described in this thesis failed to detect these lysosomal and late endosomal markers close to laser-induced membrane wounds. Whether these markers are lost during the recruitment of intracellular vesicles or whether vesicles positive for these markers are not involved in the response to this sort of wounding is not yet clear.

In this study we show that all ESCRT-III proteins with the exception of CHMP6 (known as Vps20 in yeast) localize to membrane wounds. This result bears a striking resemblance to both cytokinesis and virus budding where ESCRT-II and CHMP6 are also unnecessary [von Schwedler 2003, Langelier 2006]. CHMP6 links ESCRT-II and ESCRT-III complexes by interacting with both Vps25 (ESCRT-II) and Snf7/CHMP4 (ESCRT-III) [Teo 2004, Teis 2008, Teis 2010]. Therefore, it is unsurprising that the absence of requirement for the ESCRT-II complex is accompanied by the absence of requirement of CHMP6. Moreover, the role of ESCRT-II and CHMP6 is restricted to mammaliam ILV formation [Bowers 2006, Langelier 2006, Malerod 2007]. This indicates that ESCRT functions in other organisms can bypass these proteins, whilst conserving a fully functional ESCRT machinery. The finding that yet another function of ESCRT (its role in plasma membrane repair) follows the same principle may suggest that the function of ESCRT-II and its associated ESCRT-III protein CHMP6 are entirely restricted to ILV formation.

Moreover, we could see a clear involvement of the protein ALIX during plasma membrane repair: it is possible that ALIX recruits ESCRT-III proteins to the damaged plasma membrane replacing the canonical mechanism described during ILV formation where ESCRT subcomplexes are believed to be recruited in a sequential fashion from ESCRT-0 to ESCRT-III. Indeed, ALIX has been proposed to be an adaptor protein able to recruit the ESCRT-III machinery independently of ESCRT-0, I and II during virus budding and probably also during cytokinesis. Consistently, TSG101 is essential for the budding of arenavirus and paramyxovirus [Perez 2003, Schmitt 2005], but seems to be dispensable for HIV-1 budding because abolishing the interaction of TSG101 with the viral Gag protein can be efficiently rescued by ALIX [Usami 2007]. The presence of ALIX in plasma membrane wounds suggests a possible replacement of some ESCRT subcomplexes, particularly the ESCRT-0 machinery including HRS, which was not detected at the wound site. The requirement of TSG101 in the response to wounding has still to be confirmed but our data suggest that TSG101 may be present at the wound sites. Moreover, the presence of ubiquitin at the wounding site strengthens the probability that an adaptor between ubiquitination and ESCRTs may exist, the best candidates being HRS and TSG101.

4.1.2 ESCRT selective recruitment

Previous studies have shown that the presence of an oxidative environment such as the extracellular medium seems to be important for wound repair. Indeed the oligomerization and the translocation of MG53 at the wounding site is dependent on its oxidation. Oxidation may constitute a much broader signal for cell damage,

including wounding. Indeed, although still debated, studies suggest that oxidized proteins could be ubiquitinated [Shang 2011]. Ubiquitin enrichment could also be induced by laser-mediated damage of proteins. Therefore, upon protein damage, rapid ubiquitination could mediate the recruitment of machinery for membrane wound repair. In support of this hypothesis, some pathogenic mutant forms of proteins involved in plasma membrane maintenance are ubiquitinated and their targeting towards the plasma membrane is impaired. This is the case for Caveolin-3 and alpha-Sarcoglycan [Gastaldello 2008, Galbiati 2000b]. It is therefore possible that the oxidation of these proteins could also cause some sort of mis-folding that could trigger their ubiquitination and the subsequent recruitment of repair machineries. Further experiments on ESCRT recruitment to the wound site in the presence of anti-oxidants would be an interesting method of testing this hypothesis. The observation of Ubiquitin at the wound site could also indicate the implication of other Ubiquitin-related mechanisms such as mechanisms involving the proteasome, autophagic machinery or Ubiquitin-mediated signaling, although autophagic machinery do not appear to be involved as no reaction of the protein LC3 was seen during laser-wounding of the plasma membrane.

Other mechanisms for ESCRT recruitment are also possible. Our data particularly show an interesting enrichment of CHMP4B on the membranes of blebs that form after plasma membrane wounding. Besides their rounder shape, growing blebs are characterized by their very low membrane tension approximately 2-fold lower than the normal plasma membrane tension [Dai 1999]. This lowered membrane tension is correlated with a diminished binding to the cytoskeleton. When considered together, these characteristics may be indicators for a more physical model for ESCRT recruitment. Indeed, it has been shown *in vitro* by [Wollert 2009] that ESCRT-II has the capacity to induce membrane invagination prior to the recruitment of ESCRT-III. It is therefore possible that ESCRT-III sub-complex is preferentially recruited to membranes with special curvature. It is also likely that ESCRTs are recruitebetad specifically to membranes with low tension such as growing blebs. Exocytosis [Steinhardt 1994, Bi 1995, Miyake 1995, Bi 1997, Togo 1999, Huynh 2004, Tam 2010, Hagmann 1992, Dai 1997, Huynh 2004]and actin depolymerization [?, Miyake 2001, Togo 2000] that are characteristic of plasma membrane repair, and which contribute to lower membrane tension surrounding wounds, may promote not only spontaneous resealing of small wounds but also ESCRT recruitment for the resealing of small wounds that does not reseal spontaneously. Consequently, lowering membrane tension *via* exocytosis or actin depolymerization, may help to reseal plasma membrane holes spontaneously and may accelerate healing *via* the recruitment of ESCRT proteins[1]. The capacity of ESCRT subunits to directly bind lipids directly and, most importantly, the affinity of some ESCRT subunits for lipids specific to the plasma membrane are also arguments for the direct recruitment of ESCRT-III proteins to the plasma membrane. Indeed, overexpressed ESCRT-III proteins have been found on the plasma membrane in spiral -shaped filaments or pics [Hanson 2008, Bodon 2011].

Finally, dystroglycan loss of function has been associated with sarcolemma instability [Kabaeva 2011]. Interestingly, beta-dystroglycan has a PPXY motif

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1. Note that a dense actin cytoskeleton attaching to the membrane may also avoid proper ESCRT polymerization by steric hidrance

[Sotgia 2000]. This kind of motifs have been involved in the recruitment of TSG101 [Perez 2003, Schmitt 2005] and ESCRT-III proteins by virus proteins *via* ALIX binding [von Schwedler 2003, Fisher 2007, McCullough 2008, Strack 2003, Usami 2007, Zhai 2008]. It is therefore possible that ESCRTs are recruited to plasma membrane wounds by proteins containing PPXY motifs that are also necessary for plasma membrane integrity such as dystroglycans.

4.1.3 Reconciliation of previous models for membrane repair with the ESCRT-mediated pinching model
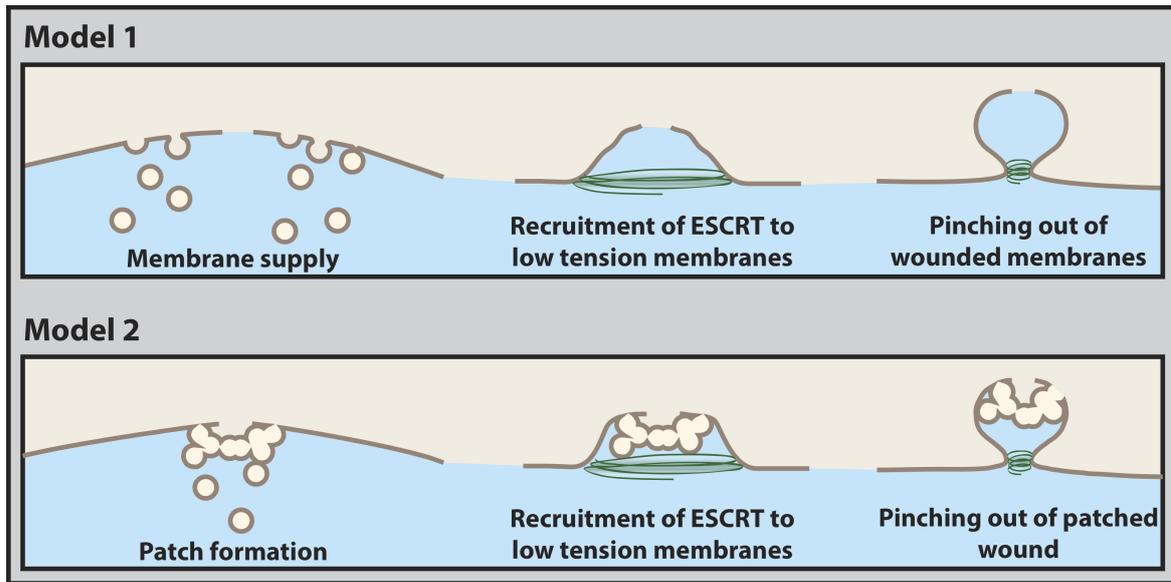
FIGURE 4.2 – **Reconciliation between previous models and ESCRT pinching model**

The ESCRT-mediated pinching model that we propose could explain the repair of a variety of wounds by itself. Nevertheles, this model is not at all incompatible with other models of membrane repair and it is very likely that it occurs in synergy with other phenomenons observed during this process.

As I discussed previously, ESCRT could be recruited to membrane growing blebs, that is, to low tension membranes. Previous studies have shown that lower tensions favor membrane resealing in simple systems such as liposomes [Taupin 1975, Zhelev 1993, Moroz 1997, Sandre 1999]. Additionally, several studies have demonstrated the necessity of exocytosis and clearance of the cytoskeleton during membrane repair [Steinhardt 1994, Bi 1995, Miyake 1995, Bi 1997, Togo 1999, Huynh 2004, Tam 2010, Hagmann 1992, Dai 1997, Huynh 2004, Togo 2000, **?**, Miyake 2001], both that are susceptible to lower the plasma membrane tension [Raucher 1999, Dai 1997]. Taken together, these observations support a model whereby a controlled and local decrease in membrane tension favors the recruitment of ESCRT subunits in a physical-based manner. The fact that both ESCRTs devoid of the auto-inhibition domain [Hanson 2008]

and highly over-expressed ESCRT subunits [Bodon 2011] bind to the plasma membrane could indicate that even at low expression levels some ESCRT subunits localize to the plasma membrane. This, for example, is compatible with the fact that CHMP3 is able to bind PtdIns(3,4)P_2 which is produced mainly at the plasma membrane [Whitley 2003].

Hence, it is possible that some ESCRT subunits remain at the plasma membrane as a sort of "nucleators" for ESCRT-III assembly upon a specific signals including: oxidation or ubiquitination of proteins, exposure of PPXY motifs, or allosteric clearance of the wound sites. Another important fact is that most vesicles undergoing exocytosis during plasma membrane repair are likely to be lysosomes [Miyake 1995, Tam 2010], endosomes [Miyake 1995, **?**] or Golgi derived secretory granules [**?**]. Besides their role as membrane suppliers, these compartments can also act as membrane composition modifiers by recruiting lipids that facilitate or reinforce the binding of proteins important for membrane resealing, such as ESCRTs. See Figure 5.2 - Model 1 for visual comprehension of the model reconciling the exocytosis requirement during plasma membrane repair with ESCRT-mediated pinching of damaged portions of membrane. Note that endosomal/lysosomal fusion is not observed in the wound area before the accumulation of ESCRT, hence is seems unlikely that endosomes/lysosomes directly deliver ESCRT proteins to the wound, these proteins being rather recruited from a cytosolic pool.

Besides, although the presence of a vesicular patches has been demonstrated for very large wounds in oocytes or eggs of invertebrates, no data exists for vesicular patching of smaller wounds. It is clear that vesicles approach the plasma membrane upon wounding, but whether they form a patch or perform another function such as lowering local membrane tension or delivering specific lipids has not been fully elucidated. Counter-arguments against the patch hypothesis for small wounds include the fact that vesicles recruited at the wounding site are not restricted to the area predicted to correspond to the site of membrane damage but rather are recruited to a larger area of the plasma membrane, as seen in experiments from Cai and co-workers [Cai 2009]. This is of course not incompatible with a double role for exocytosis in simultaneously patching and lowering the tension of membrane. Another counter-argument stated by Idone and co-workers [Idone 2008b, Idone 2008a] is the fact that "stable" wounds made by pore-forming proteins cannot be sealed by patches. However, patch mediated removal of pore-forming molecules remains a logical and conceivable hypothesis. The patch hypothesis for small wounds is also be reconcilable with the ESCRT-mediated budding in a model whereby rapid patching of wounds in the plasma membrane is followed by ESCRT budding-mediated removal of these patches, which are probably discordant in homogeneity and composition with the plasma membrane. See Figure 5.2 Model 2 for visual explanation.

Another model which has been proposed for the elimination of pore-forming proteins by endocytosis, is one in which the pore-inducing proteins would be degraded through ES-CRT dependent internalization of endosomes [Tam 2010, Idone 2008b, Corrotte 2012]. However, the model proposing the budding of lesions seem more likely at the level of a single cell. Indeed, even if pore forming proteins can be degraded by the cell machinery, they still must undergo proper endocytosis before reaching the MVBs. The deficiency

with this hypothesis is that endosomes transporting pore-forming proteins from the plasma membrane to the MVBs may themselves be perforated by the pore-forming toxins. Studies presented in Chapter 3 together with our results, suggest that endosomes with altered properties, possibly including disrupted membranes, are likely to be targeted by the cell´ s autophagic machinery. It is very doubtful that these modified endosomes can still traffic normally to MVBs. Nevertheless, it is still possible that the involvement of ESCRTs in the elimination of pore-forming proteins involves a role for ESCRTs in autophagy [Corrotte 2012]. Moreover, pore forming non-proteic molecules are likely to be very difficult for lysosomes to degrade. Their internalization would then mean the perpetuation of a wound in intracellular membranes. An extracellular budding-mediated elimination is more coherent with a "selfish cell" theory in which the externalization of pore-forming molecules that perturb neighbor cells is preferred over loss of an individual cell.

The detection of cell damage by wounded cells is crucial for the resealing process to occur and the avoidance of cell death. The process of detection is likely to involve the sensing of the extracellular environment conditions such as Ca^{2+} concentration. The release of cytoplasmic constituents such as FGF2 by observed in endothelial cells, for example, [McNeil 1989b, Muthukrishnan 1991] allows a tissue-level response for the replacement of damaged cells and is crucial for tissue maintenance and renewal. Wound-sensing may also constitute a signal for mechanical stress at the tissue or cell population level [Grembowicz 1999]. As stated previously, at the whole organism level, the budding and release of small vesicles containing pore forming molecules could also represent an important signal for the immune system response: these vesicles could not only present the pathogenic molecules themselves but also their membrane bound versions.

4.1.4 Paralellels between the membrane repair role of ESCRTs and other known functions

The role of the ESCRTs during plasma membrane repair appears to more closely ressemble their role in cytokinesis and virus budding than their role in ILV formation in terms of the proteins involved. The topology of the buds observed after wounding of the plasma membrane is similar to that observed during virus budding. In both cases the buds are similar in size. Note that the clusters of buds towards the extracellular medium similar to those seen in this work have been observed for caveolae (this time towards the cytoplasm) [Parton 2007].

Besides the requirement of the ESCRTs for pathways such as ILV biogenesis and cytokinesis, other machineries are also common to these two cellular events and plasma membrane repair. A very striking similarity is the fact that both endocytosis and exocytosis are crucial for both cytokinesis and plasma membrane repair. [Steinhardt 1994, Bi 1995, Miyake 1995, Bi 1997, Togo 1999, Togo 2000, Huynh 2004, Tam 2010, Idone 2008b, Tam 2010, Corrotte 2012, Thiery 2010, Danilchik 1998, Gerald 2001, Henne 2011, Echard 2008, Montagnac 2008]. In both cases, it is very likely that exocytosis is an important factor in membrane supply, either by allowing

the membrane to deform more easily, which is consistent with a decrease in membrane tension, or by changing the lipid composition of the membrane as was show for cytokinesis where the enrichment of PdtIns(4,5)P$_2$ at the bridge is required for cytokinesis of different species. For a review of this topic see [Montagnac 2008, Echard 2008]. In plants, the trafficking of vesicles from the Golgi apparatus is crucial for the formation of the cell plate in a special exocytic event whereby vesicles fuse with each other prior to fusing with the plasma membrane. This closure event could be compared with the resealing of a very large hole. Besides, it is possible that endocytosis occurs in both processes, in part to compensate for the increased amount of membrane brought by exocytosis. In the case of the plasma membrane repair, this is compatible with the fact that exocytosis is necessary for and occurs prior to endocytosis [Tam 2010]. During cytokinesis, it is also likely that endocytosis contributes to the removal of membrane at the bridge in order to reduce its diameter. This process may also compensate for the changed lipid composition induced by massive exocytosis. Another possibility is that endocytosis ensures the recycling of an excess of certain proteins transported to the membrane by exocytosis. Another curious similarity is the presence of Phosphatidyl-Ethanolamine in the outer leaflet of the membrane during cytokinesis and of Phosphatidyl-Serine during membrane wounding. Both lipids are usually located at the inner leaflet thanks to active translocation by P4 ATPases. See for reviews [Echard 2008, Draeger 2011, van Meer 2008]. All these similarities let us think that not only cytokinesis and membrane repair may be similar events, but also that during cytokinesis, micro-wounding events may take place at the cellular bridge. For example, the pulling of the daughter cells may damage the bridge or an external "helper" cell may do so. Besides, in both plasma membrane repair and cytokinesis, the SNAREs have been implicated. Their role in both mechanisms would be to mediate homotypic vesicle fusion or vesicle fusion with the plasma membrane during exocytosis. We cannot exclude that these proteins could work in synergy with ESCRTs during membrane fission/fusion events.

Another important observation is the involvement of Annexins during membrane repair (AnnexinA1), cytokinesis (AnnexinA11) and ILV biogenesis (AnnexinA2) [Tomas 2004, White 2006, Morel 2009]. Proteins of the Annexin family bind both Ca^{2+} and phospholipids, which make them excellent candidates for sensing both Ca^{2+} and membrane lipid composition, perhaps allowing the recruitment of specific machineries on membranes. Some of them are able to drive vesicle transport and vesicle fusion, which may mean they could also be involved in exocytosis at least during membrane repair and cytokinesis.

Further experiments are needed to confirm that the ESCRT-0, I and II subcomplexes are not involved in ESCRT-dependent budding during plasma membrane repair. Epistasy experiments would be very interesting to position the role of ESCRTs among all other membrane repair machineries described previously. These experiments should help us to further decide about the different possible models for ESCRTs job in this cellular mechanism. Additional electron-microscopy experiments should help us to determine the shape taken by ESCRT-III proteins during this process. Because our system is safe and easy to perform, we believe that it represent an interesting set-up to

further explore the mechanism of ESCRTs function, in addition to cytokinesis and virus budding. Further experiments are being performed to understand a possible relationship between ESCRT mediated cellular functions, in particular between cytokinesis and plasma membrane repair. Although the first role ascribed to ESCRTs was in MVB biogenesis, it is unlikely that this represents the first function that appeared during evolution. Indeed, MVBs does not exist in Bacteria or Archaea, and yet these organisms undergo cell division. Moreover, as we saw before, ESCRT homologs are required for Crenarchea cell division [Lindas 2008, Samson 2008]. Besides, extracellular vesicles are generated by all Bacteria, Eukaryotes and Archaea in an ESCRTs-homologues dependent manner. See [Deatherage 2012] for review. Interestingly, a hypothesis for the origin of cell division is the bud formation. This hypothesis is supported by cell-division mechanisms such as the one from budding yeast, which suggests that the origin of ESCRT functions is the formation of the extracellular bud.

4.2 Dealing with organelle damage.

Organelle damage may result from a vast range of different causes including pH changes,redox conditions, drugs, heavy metals and free radicals. Harmful proteins such as pore-forming toxins or misfolded proteins can also alter organelles. Mis-folded and aggregated proteins affect, for example, organelle function and can perturb the luminal activity or interact with membranes, or even create pore-like structures within actual membranes.

4.2.1 The importance of dealing with damaged trafficking organelles

As seen in Chapter 1, most organelles are linked and depend upon each other for their biogenesis and homeostasis. Organelles define compartments and are isolated from the extracellular space and are in constant communication with their immediate environment. Organelle integrity can be affected by several means. For example, toxin inserted at the level of the plasma membrane can be endocytosed and create endosomal wounds. This may be the case for *Vibrio cholera* cytolysin which, on occassion, induces the formation of large cytoplasmic vacuoles in intestine epithelial cells which, in turn, sometimes induces cell death [Coelho 2000, Figueroa-Arredondo 2001, Gutierrez 2007]. These large vacuoles of endosomal or lysosomal origin have been observed for other pore-forming toxins such as VacA produced by *Helicobacter pylori*, which also make pores in mitochondria. See for review [Rassow 2011]. Listeriolysin O which forms a pore in a pH dependent manner, and therefore permeabilizes membranes only when it is internalized in endosomes [Geoffroy 1987]. The aerolysin pore forming toxin, produced by *Aeromonas hydrophila*, triggers vacuolation of the endoplasmic reticulum [Abrami 1998]. Whether this vacuolation comes from the permeabilization of the ER or from side effects of the permeabilization of the plasma membrane remains unclear. Besides pore-forming toxins, other pathogen machinery can perturb organelles. Certain bacteria such as *Salmonella* are able to enter into the cell hijacking the endocytosis

mechanisms of the host and are found in endosomes called *Salmonella*-containing vacuoles (SCV) [Lostroh 2001]. Once in the cytoplasm, some *Salmonella* cells are able to escape from the SCV by inducing lysis of the membrane that surrounds them [Brumell 2002]. Additionally, it has been shown that activation of the Nalp3-inflammasome, which in turn is involved in the activation of caspase-1, is triggered by the leaking of lysosomal enzymes into the cytoplasm. This shows that the leaking of mitochondria is not the only organelle leak that is extremely toxic for the cell [Hornung 2008].

Although complications with pore-forming toxins have not been described for the Golgi apparatus, some congenital disorders (Congenital Disorders of Glycosylation or CDG) have been associated with defects in the Golgi apparatus function caused by mutated glycosylation enzymes. Intriguingly, these genetic diseases have been associated with mutations in very different proteins including mutations in SEC23B (a component of the COPII coat [2]), mutations in the COG complex [3] and mutations in ATP6V0A2 (a subunit of the vacuolar ATPase, which is responsible for the acidification of several membrane bound organelles such as endosomes vacuoles, lysosomes, and the Golgi apparatus and its related compartments). See for review on glycosylation disorders [Rosnoblet 2012]. The effect of mutations in ATP6V0A2 on glycosylation is likely to rely on the perturbation of the Golgi apparatus pH. The Golgi pH regulator channel (GPHR) has been identified, as its name indicates, as a channel responsible for the regulation of the Golgi pH. This protein seems to be more specific to the Golgi apparatus and the Trans-Golgi network than the vacuolar H^+-ATPase and its disruption triggers morphological aberrations of the Golgi apparatus as well as a delayed trafficking and impaired glycosylation [Maeda 2008]. Additionally, toxic molecules like Monensin [4], which acts as a Na^+/H^+ exchanger, cause a pH increase and swelling of the Golgi apparatus that leads to blocking of secretion. Similarly, Bafilomycin which inhibits vacuolar H^+-ATPase perturbs Golgi function and morphology. Other molecules like Okadaic acid trigger morphologic alteration in the Golgi morphology which are only partially reversible. See for review [Dinter 1998]. Moreover, the Rose Bengal acetate, drug used for photodynamic anti-cancer therapy accumulate preferentially at the Golgi apparatus [Soldani 2004]. It would be interesting to test whether Golgi damage is involved in cell death in this case. In our experiments, cells depleted for NBR1, in addition to being more prone to cell death for unknown reasons, are also very sensitive to Golgi damage by DAB intoxication. The localization of NBR1 at the damaged Golgi apparatus could thus suggest an important protective role of NBR1 during Golgi damage. All the examples cited above suggest that Golgi damage may be triggered by different means and show also the importance of Golgi homeostasis, and in general, of trafficking organelle homeostasis. This strengthens the importance of understanding the mechanisms involved in dealing with damaged trafficking organelles.

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2. COPII is the coat of vesicles trafficking from the ER to the Golgi apparatus.

3. As a reminder, the COG complex, or Conserved Oligomeric Golgi complex, is thought to play a role in intra-Golgi retrograde trafficking.

4. Monensin is produced by *Streptolyces cinnamonensis*. It is used as an antibiotic in animal food but some cases of food poisonning of equines due to Monensin have been reported, which reveal their toxicity for mammals.

4.2.2 Arguments for damaged Golgi elimination by autophagic machinery.

The first step in macroautophagy and related selective mechanisms corresponds to the surrounding of the target cytoplasmic fraction, aggregate, organelle or pathogen by an autophagosomal membrane. In the case of potentially dangerous compounds such as intracellular pathogens or leaking and non-functional organelles, this isolation step may represent the most crucial part of the process. This isolation can potentially avoid the leak of Cytochrome C from permeabilized mitochondria for example, which may lead to cell death by apoptosis. It can also avoid the interaction of protein machinery with damaged and non-functional organelles, avoiding therefore aberrant signaling and processes. Damaged Golgi apparatuses could, for example, continue to receive cargo through retrograde and anterograde trafficking. S. Bernales and co-workers have shown that during the ER expansion phase of the Unfolded Protein Response, isolation without degradation of the ER by autophagy is sufficient to protect cells.

Some studies suggest that the Golgi apparatus and related compartments can be eliminated through autophagy. In the mosquito *Aedes aegypti*, Golgi complexes have been detected in lysosomes, most likely delivered by autophagic vesicles, during cellular remodeling [Raikhel 1986, Mijaljica 2006]. Other studies have also reported selective degradation of the Golgi apparatus in butterfly larvae and the tobacco hornworm *Manduca sexta* [Locke 1975, Willott 1988]. See for review [Mijaljica 2006]. Golgi derivatives were reported in autophagosomes, although in very low levels in rat hepatocytes [Marzella 1982]. Aggregates of Apoprotein-B exiting from the Golgi have been shown to be targeted by autophagy [Pan 2008]. The potential elimination of the Golgi apparatus is supported by recent studies, and one from my laboratory, showing that the cell can probably form a new Golgi apparatus following the inactivation or removal of an existing Golgi apparatus [Jollivet 2007, Tangemo 2011].

This is an important point because, unlike other organelles such as mitochondria, peroxisomes or endosomes, which exist in multiples, many cells present one functional Golgi apparatus unit. Whether this functional unit corresponds to several very close Golgi apparatuses or if this unit is fully connected is not yet clear. Nevertheless, our experiments using a 2-photon laser to specifically damage the Golgi apparatus demonstrated recruitment of LC3 to the entire MannosidaseII-mCherry-labelled Golgi unit. This strongly suggests that by damaging a very localized portion of the organelle, the whole Golgi apparatus is damaged, probably because it constitutes one only fully connected organelle.

Moreover, as stated above, *Samonella* can trigger the lysis of the SCV after it is internalized by the cell. It has been shown that these SCV can be surrounded by autophagosomes. In the absence of Atg9L1 which is required for autophagosome formation, SCV membranes are directly labelled by LC3 [Kageyama 2011], suggesting that the damaged membranes of trafficking organelles can be directly labelled by LC3. Our work shows that endosomes can be targeted by autophagic machineries upon DAB intoxication, supporting the idea that SCVs are targeted by autophagy because they are detected as damaged organelles, probably upon *Salmonella*-triggered lysis. Our results on the damaged Golgi apparatus suggest strongly that the membranes of this

organelle can be directly targeted by LC3. The same may hold for endosomes and related compartments such as SCV and may explain why SCVs are still targeted by LC3 even in the absence of functional autophagosome formation. Analyses performed on SCVs are also in agreement with our observations of damaged Golgi being directly labelled by LC3. It is possible that LC3 plays a double role in the elimination of damaged trafficking organelles. One role would be to label the damaged membranes of trafficking organelles, and perhaps recruit other proteins such as p62 to favor ubiquitination and recruitment of autophagosomal membranes. Another role would consist of ensuring autophagosome expansion and closure. The timing of LC3 recruitment to the damaged Golgi in our work appears to occur prior to the enrichment in p62 and ubiquitin. Moreover, p62 is not necessary for LC3 recruitment at the damaged Golgi apparatus, and it is the case also during mitophagy [Okatsu 2010, Narendra 2010]. Whether this is due to the presence of other autophagy receptors like NBR1 is not yet known, but our results suggest a hierarchical order of events because disruption of LC3 recruitment by expression of an Atg4 dominant negative construct seems to perturb both ubiquitin and p62 enrichment at the damaged Golgi apparatus.

Besides, the Golgi apparatus appears to play an important role for autophagosome formation. This may explain only limited cases in which damaged Golgi apparatus was detected inside LC3 positive vacuoles. It is possible that the engulfment of the damaged Golgi apparatus occurs once the cell has reformed a fully functional Golgi apparatus, capable of supplying *de novo* formed autophagosomal membranes. The contribution of organelles like the Golgi apparatus to autophagosome formation may also explain why their membranes can be directly labelled by LC3. It is possible that damaging in the Golgi apparatus trigger the same signal as that for autophagosome formation. The signal that labels the Golgi apparatus as damaged could be for example the loss of lipid assymetry on the Golgi membranes. Other potential signals could include the pioneer ubiquitination of proteins. Indeed, we saw that ubiquitination can happen very quickly at the wounded plasma membrane. It is therefore possible that it is a very rapid cellular response for damaged organelles also. Another possible signal could be the phosphorylation of proteins at the surface of the Golgi apparatus or other modifications. Indeed, phosphorylation has been proposed as a sensing signal for the massive arrival of proteins to the Golgi apparatus [Pulvirenti 2008]. Although EM in this study shows that the Golgi apparatuses enriched in phosphorylated proteins present a slightly swollen morphology, it is not clear if this massive arrival of proteins to the Golgi apparatus perturbs its homeostasis. In this context I studied the effect of a massive arrival of proteins to the Golgi using thermo-sensitive VSV-G and the RUSH-system (recently published by our laboratory). However these massive trafficking events failed to induce clearly detectable LC3 recruitment at the Golgi [Lafay 1974, Kreis 1986, Boncompain 2012]. Nevertheless, experiments over-expressing MannosidaseII-mCherry construct did induce the recruitment of LC3 when over-expression levels were very high. Intriguingly, the incubation of cells with Brefeldin A after DAB precipitate intoxication of the Golgi apparatus inhibited LC3 recruitment.

Finally, it is of note that recent studies have shown the existence of exocytic events mediated by autophagic machineries that also involve the Vps23/TSG101 protein

[Bruns 2011], which, as we saw before, is an ESCRT-I protein. This suggests that even damage to intracellular membranes could be potentially externalized in an ESCRT-dependent manner. Whether these secreted autophagic compartments are involved in the elimination of damaged organelles is yet to be elucidated. Future experiments may attempt to further understand the primary signal that labels the Golgi apparatus and endosomes as damaged. Screens of kinases, Ubiquitin-ligases or other proteins involved in post-translational modifications of proteins should be explored. Other proteins involved in the establishment of lipid asymmetry should also be considered. Drugs that inhibit the targeting of trafficking organelles by autophagic machinery should be considered to make cells more sensitive to certain treatments that act through the damage of these organelles.

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