

Role of Confinement on Clathrin-mediated Endocytosis Dahiana Le Devedec

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Role of confinement on clathrin-mediated endocytosis

Thèse de doctorat de l'Université Paris-Saclay préparée à l'Université Paris-Sud

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Thèse présentée et soutenue à Villejuif, le 17 septembre 2019, par

Dahiana Le Dévédec

Président

Composition du Jury :

Thomas Mercher DR, Gustave Roussy (– UMR 1170)

Danijela Vignjevic DR, Institut Curie (– UMR 144)

Cédric Delevoye CR, Institut Curie (– UMR 144)

Mark Scott DR, Institut Cochin (– U1016)

Guillaume Montagnac DR, Gustave Roussy (– UMR 1170) Rapporteur Rapporteur Examinateur

Directeur de thèse

"Life is either a daring adventure, or nothing."

Helen Keller

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ABBREVIATIONS

ADAM

a disintegrin and metalloproteinase

AFM

Atomic force microscopy

AMT

amoeboid/mesenchymal transition

ANTH

AP180 N-terminal homology

AP

adaptor protein

AP2

Adaptor Protein 2

AREG

amphiregulin

ARH

autosomal recessive hypercholesterolemia

BAR

Bin, Amphiphysin, Rvs

BTC

betacellulin

CAF

cancer-associated fibroblast

CALM

Clathrin assembly lymphoid myeloid leukemia

ССР

clathrin-coated pit

CCS

clathrin-coated structure

CCV

clathrin-coated vesicle

CHC

clathrin heavy chain

CLC

clathrin light chain CLEM Correlative light-electron microscopy CLIC clathrin- and dynamin-independent carrier CME clathrin-mediated endocytosis Dab2 disabled homolog 2 ECM extracellular matrix EE early endosome EGF epidermal growth factor EGFR epidermal growth factor receptor EMT epithelial-mesenchymal transition **ENTH** epsin N-terminal homology EPI epigen Eps15 EGF receptor phosphorylation substrate 15 ERC recycling endosome compartment EREG epiregulin FA focal adhesion

FAK

focal adhesion kinase

FCHo

F-BAR domain-containing Fer/Cip4 homology domain-only

FEME

fast endophilin-mediated endocytosis

FH

familial hypercholesterolaemia

FRAP

fluorescence recovery after photobleaching

GAK

cyclin G-associated kinase

GEEC

GPI-AP- enriched early endosomal compartment GPCR

G protein-coupled receptor

HB-EGF

heparin-binding EGF-like growth factor

HNSCC

head and neck squamous cell carcinoma

Hrs

hepatocyte growth-factor regulated tyrosinekinase substrate

Hsc70

heat shock cognate 70

ILV

intraluminal vesicle

JNK

c-Jun N-terminal kinase

LDL

low density lipoprotein

LDLR

low density lipoprotein receptor LECA

Last Eukaryotic Common Ancester LOX lysyl oxidase MAPK mitogen activated protein kinase MAT mesenchymal/amoeboid transition MMP matrix metalloproteinase **MVBs** multivesicular bodies NECAP adaptin ear binding coat associated protein NSCLC non-small-cell lung cancer PH Pleckstrin Homology PI(4,5)P2 phosphatidylinositol 4,5-bisphosphate PI3K phosphatidyl-inositol-3-kinase PIP2 phosphatidylinositol (4,5)- bisphosphate PM plasma membrane PTB domain phosphotyrosine binding domain PTEN phosphatase and tensin homolog pΥ phosphotyrosine Rab4 Ras-associated binding 4 RTK receptor tyrosine kinases

SH2

Src homology domain

SNX9

sorting nexin-9

TFR

transferrin receptor

$TGF-\alpha$

transforming growth factor $\boldsymbol{\alpha}$

TGN

trans-Golgi Network

TKI

tyrosine kinase inhibitor

UIM

ubiquitin interacting motif

WGA

wheat germ agglutinin

YAP

Yes-associated protein

INTRODUCTION

I. Endocytosis

A. Historical perspective of clathrin-mediated endocytosis

Endocytosis as a crucial system which contributed to the evolution of eukaryotic cells from prokaryotic organisms is well acknowledged by the scientific community. The endosymbiotic theory was first submitted by Konstantin Mereschkowski in 1905¹ and Lynn Margulis provided the first experimental evidence of it several decades later. Based on cytological, biochemical and paleontological data, she substantiated the endosymbiotic theory to explain the prokaryotic origins of the intracellular organelles such as mitochondria and plastids². With the advance of biological techniques, biologists became more aware of the complexity of the cell and achieved major breakthroughs concerning the understanding of the cell.

Endocytosis is the broad term to design all the processes involved in the uptake of extracellular material into the cell. At the very beginning, endocytosis was nominated as cell "eating" and "drinking". The first major observation of this phenomenon was made in the early 1880's by E. Metchnikoff who discovered cells involved in the response to pathogens with the ability to eat, which he would call phagocytes from the greek term "phagos" (to eat) and "cyte" (cell)³. His work on phagocytic immunity led him to be awarded the Nobel Prize in Physiology and Medicine in 1908, which he shared with P. Ehrlich, who complemented the previous work about innate immunity with acquire immunity, for their contribution to immunology. The term pinocytosis from "píno" (to drink) was proposed later by Lewis in 1931⁴. The phagocytic theory was the prelude to the field of immunology which led to endocytosis. Beyond these pioneering studies, researchers, with the evolution of microscopy and especially electron microscopy, have made breakthrough discoveries in cell biology. It allowed them to identify every organelles in the eukaryotic cells and to determine their functions which will earn G. Palade, A. Claude and C. de Duve the Nobel Prize in Physiology and Medicine in 1974. Among these organelles, the

most relevant in the endocytic pathway are lysosomes^{5,6}. After performing tissue fractionation, different states of enzyme activity were observed. Further studies unveiled a novel enclosed organelle, the lysosome, with digestive properties and characterized by its acidic pH and its content of various hydrolytic enzymes and acid phosphatase⁷. Lysosomes are now recognized as the terminal degradative compartment of the endocytic pathway⁸.

The first description of coated vesicles was made by Roth and Porter, who studied the uptake of yolk proteins by mosquito oocytes⁹. They observed, right under the plasma membrane, invaginations (which they termed "pits") and vesicles with distinctive bristle coats.



Figure 1: Schematic representation of yolk trafficking in mosquito oocyte

This image is a hand-drawn summary of observations of endocytosis made by electron microscopy as presented by Roth and Porter. (1) depicts the first stage of invagination in the plasma membrane, (2) represents the fully developed coated pit and (3) the coated vesicle. The step (4) illustrates the vesicle uncoating which then fuses with other vesicles (5). The formation of tubules from small droplets (7) is frequently observed. Finally, larger droplets (6) coalesce to form the large crystalline protein yolk bodies of the oocyte (8).

Source: Roth and Porter, 1964⁹

From these observations, they proposed that coated pits would mature, bud off the plasma membrane and give rise to coated vesicles (Figure 1). They even proposed, after budding in the cytosol, the occurrence of an uncoating reaction at these vesicles, allowing the now naked vesicles to fuse together to form endosomes. From there, they accurately postulated this was a transient event which might contribute to the selective internalization of membrane bound material. These vesicles were then isolated from pig brain by Kadota & Kanaseki who described the coat architecture as a polygonal "basketwork"¹⁰. Years later, the first biochemical analysis of these coated vesicles was performed by Barbara Pearse^{11,12} and revealed a protein of 180kDa as the major component of these vesicles. She named it "clathrin" in reference to its lattice-like structure. Further analysis on clathrin-coated structures revealed an organization in triskelion consisting in a three legs structure which results from the assembly of three heavy chains and three light chains of clathrin^{13,14}. Since then, the coated vesicles discovered by Roth and Porter were called clathrin-coated vesicles also revealed the presence of other proteins, called adaptor proteins (AP), which promote the assembly of clathrin triskelia into clathrin coats^{15,16}.

In addition to the characterization of clathrin vesicles, Brown & Goldstein¹⁷ validated the speculation of Porter & Roth regarding clathrin-mediated endocytosis specificity. Indeed, they determined, by labelling the low density lipoproteins (LDL) with ferritin, that LDL is mostly internalized by clathrin-coated pits (CCPs). Moreover, to assess the selectivity of CME, they conducted a study on a patient affected by familial hypercholesterolaemia (FH) who displayed normal LDL binding at the plasma membrane via its receptor but no uptake and degradation. The origin of this defect was the localization of the LDL receptor (LDLR) which was randomly distributed at the cell surface instead of being clustered at the CCPs. Following this first identification of a receptor specifically internalized by CCPs, other receptors where shown to be internalized by CCPs such as the epidermal growth factor receptor (EGFR)^{18,19} and the transferrin receptor (TFR)²⁰. Over the years, research achieved by many labs has revealed the molecular details of clathrin-mediated endocytosis (CME) from how they select cargoes (the receptor and its bound ligand) to their fate²¹. Nowadays, clathrin-mediated endocytosis is

recognized as the major endocytic route for the internalization of a vast myriad of cargoes into the cell.

B. Clathrin-independent endocytosis

Even if clathrin-mediated endocytosis is the best characterized and major pathway of extracellular material entry into the cells, alternative mechanisms can be exploited by the cell to internalize high amounts of membrane and fluids²². These pathways are actually defined because they do not require clathrin and are thus called clathrin-independent pathways (Figure 2).



Figure 2: Multiple pathways for entry into the cell

Cargoes can be internalized either by a clathrin-dependent or -independent manner. Among clathrin-independent endocytic pathways, phagocytosis is used for the internalization of large particles, whereas macropinocytosis is the specific pathway for fluids uptake. Both of these mechanisms can internalize large amounts of material and the resulting vesicle is way bigger than for other pathways. As for smaller cargoes, the processes by which they enter the cell are classified by their dependence on the GTPase dynamin and by the vesicle components. Caveolae is the major clathrin-independent pathway, and the IL2-R pathway is the only one requiring dynamin in addition to CME and caveolae. Dynamin-independent internalization includes the Arf6- or flotillin-dependent endocytosis and the GPI-AP uptake via tubular intermediates (CLIC/GEEC pathway). After uptake, all cargoes are delivered, directly or not, to early endosomes for sorting either to lysosomes for degradation or recycle at the plasma membrane.

Source: Mayor and Pagano, 2007²²

As mentioned in the historical perspectives, phagocytosis was the first identified endocytic pathway observed and is used to internalized large particles. This mechanism is actin dependent and involves the formation of cup-like membrane extensions to engulf big molecules. Phagocytosis occurs mostly in specialized phagocytic cells such as macrophages and neutrophils in which the uptake of particles is highly efficient. This mechanism has a critical role in the internalization and degradation of infectious agents and participates in the immune response, inflammation, tissue remodeling and development.

Macropinocytosis, first called pinocytosis, was renamed later to make the distinction with smaller endocytic vesicles. This pathway is an actin-based endocytic mechanism as well and is employed to absorb extensive amounts of extracellular fluids and solutes, including nutrients. Solutes taken from the extracellular space are then delivered to the endocytic compartment after vesicle fusion. Macropinocytosis can be induced by growth factors, chemokines, cytokines or pathogens. This process is ordinarily displayed by macrophages and dendritic cells but also in pathophysiological conditions including cancer²³.

Caveolae were first identified by Palade and Yamada because of their peculiar morphology, flask-shaped, observed in thin sections by electron microscopy^{24,25}. They appear as small invaginations of the plasma membrane (50-80 nm). The specific component of these structures is caveolin 1, and biochemical analysis revealed an enrichment in sphingolipids and cholesterol but also signaling proteins and glycosyl phosphatidylinositol (GPI)-anchored proteins. (GPI-APs) Caveolae-mediated endocytic process is involved in the uptake of membrane components, extracellular ligands, signaling proteins (receptor tyrosine kinases RTKs), bacterial toxins (cholera, tetanus) and some non-enveloped viruses (Simian virus 40, polyoma virus). Caveolar vesicles transfer their cargoes either to endosomes or to a distinct tubular organelle called "caveosome". Unlike clathrin-mediated endocytosis, the uptake of viruses by caveolae is very slow (half-time of 90mn). Caveosomes are specific organelles in the cytoplasm that do not exhibit markers of endosomes, lysosomes, TGN, Golgi complex, or the ER. Even more, specific CME cargoes do not aggregate in these organelles neither fluid phase markers. However,

investigating caveolar endocytosis appears to be tricky since the same endocytic cargo can be internalized by different mechanisms depending on the cell type or switch from one pathway to another under certain conditions²⁶. In addition to these previously described mechanisms, small molecules can undergo clathrin and caveolin independent endocytosis as well.

In the case of GPI-APs, the plasma membrane can form tubular invaginations which further fuse with another type of endosomal compartment, termed GPI-AP enriched early endosomal compartments (GEECs). Besides, a cholera precursor (CtxB) can be endocytosed through small tubular or ring-like carriers called clathrin- and dynamin-independent carriers (CLICs).

A new clathrin-independent process was recently uncovered and characterized by two research teams. The two studies are complementary : the first one describes and characterizes the molecular machinery of a fast clathrin-independent pathway whereas the second one focuses on the hijacking of this pathway by the bacterial Shiga and cholera toxins^{27,28}. This process is dependent of the membrane-bending protein endophilin, firstly associated to late stages of CME. The authors named this endocytic machinery fast endophilin-mediated endocytosis (FEME), due to its molecular composition and speed. They confirmed the ability of endophilin to bind activated receptors at the plasma membrane and to recruit dynamin and actin. Unlike CME, FEME occurs only for activated receptors or toxin-induced deformability, not constitutively, and at the leading edge of migrating cells. Indeed, receptor activation triggers phosphorylation of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) and forms PI(3,4,5)P3 recognized by lamellipodin at the leading edge of cells, which recruits in turn endophilin. The membrane then invaginates into tubules further release as vesicles in the cytoplasm.

It is noteworthy that eventually all routes of entry finally lead to early endosomes for further sorting.

II. Clathrin-mediated endocytosis

A. Sequential steps of CME

The process of clathrin-mediated endocytosis have been characterized through the use of biophysical, structural and biochemical approaches. Considering the presence of the major protein families involved in CME in the Last Eukaryotic Common Ancester (LECA)²⁹, endocytosis and membrane trafficking in general are well preserved mechanisms across the diversity of eukaryotes³⁰. The endocytic machinery consists of a successive and combined highly regulated key stages easily visualized by electron microscopy (Figure 3).



Figure 3: Time course of the formation of a clathrin-coated vesicle

Electron microscopic views of the different steps of clathrin-mediated endocytosis. It starts from an almost flat clathrin-coated membrane site (A) which progressively bends (B) into an homogenous curved clathrin-coated pit (C). Deeply invaginated pits display a spherical shape connected to the cell surface by a membrane neck (D). The clathrin-coated vesicle is lastly released into the cytoplasm (E). Scale bar: 200 nm.

Source: Haucke and Kozlov, 2018³¹

The cycle of a clathrin-coated vesicle is defined by four stages: initiation, maturation, scission and uncoating (Figure 4). All these steps are precisely regulated by a large diversity of proteins. Briefly, an endocytic event is initiated at the plasma membrane through the coordination of adaptors and accessory proteins. Their clustering settle the nucleation module at a precise location at the internal leaflet of the plasma membrane. This module contributes to the recruitment of more clathrin at the plasma membrane, thus stabilizing the clathrin-coated pit formation. Along with clathrin, auxiliary proteins support membrane curvature generation and stabilization to form the clathrin-coated pit. The CCP continues to expand and clathrin polymerization, with the action of other proteins, bring closer the edges of the pit by promoting the formation and constriction of the vesicle neck. The vesicle detachment takes place at the neck and requires the large GTPase dynamin which controls membrane scission. The clathrin coated vesicle is released inside the cell where disassembly proteins will promote the dismantlement of the coat. The whole cycle lasts approximately 1 minute. The naked vesicle then undergoes further trafficking and deliver its cargoes to their next destination by fusing with early endosomes. These four steps are described in details below.



Figure 4: The four steps leading to the formation of an endocytic vesicle

Clathrin-mediated endocytosis is initiated by the recognition of receptors at the plasma membrane through adaptor proteins to form nascent clathrin-coated pits (CCPs). Many endocytic accessory proteins collaborate with clathrin and adaptors for CCP growth and maturation. During late stage of CME, recruitment of dynamin help to form a collar around the neck of deeply-invaginated CCPs and drive membrane scission. The newly-formed clathrin-coated vesicle is then released into the cytosol where an uncoating reaction takes place. The naked vesicle is now able to fuse with endosomes to deliver its cargoes and the coat constituents are recycled back to the plasma membrane.

Source: adapted from Schmid, 2017³²

1. Initiation

Clathrin itself, the main component of endocytic vesicles, is unable to bind to the lipids composing the plasma membrane neither to transmembrane receptors fated for internalization.

Therefore, clathrin assembly at a specific location requires the assistance of additional proteins: adaptors and accessory proteins^{15,33,34}. These proteins have the ability to recognize and attach simultaneously membranes, specific cargoes, clathrin and other components of the endocytic pathway³⁵.

- a) Major actors
- i. Adaptor proteins

Adaptor proteins (APs) are a family of heterotetrameric complexes made of AP1, AP2, AP3, AP4 and AP5, all involved in transportation inside the cell. Their role is to bridge scaffold proteins such as clathrin to membranes as well as to recruit cargoes to be internalized. AP2 is located only at the plasma membrane whereas AP1, -3, -4 and -5 location is limited to endosomes and trans-Golgi Network (TGN)³⁶.

The Adaptor Protein 2 (AP2) is the second major component of clathrin-coated pits. AP2 is a heterotetramer consisting of four subunits: α , β 2, μ 2 and σ 2 adaptins (Figure 5).





(A) Schematic representation of AP2 complex composed of four different subunits. The core consists of the Nterminal domain of α and β 2 along with μ 2 and σ 2 subunits. The appendage domains or ears include the C-terminal domain of α and β subunits linked to the AP2 core via a flexible hinge. (B) Schematic representation of AP2 subunits protein domains. The core of the α subunit is involved in the AP2 recruitment to the plasma membrane, the μ 2 subunit is crucial for cargo recognition and the hinge of the β 2 subunit binds to clathrin. Both the appendages are able to interact with other accessory proteins and adaptors. Regarding the small σ 2 subunit, its role in purely structural and help to hold together the subunits of the AP2 complex.

Source: (A) adapted from Traub, 2009³⁷; (B) Maldonado-Báez and Wendland, 2006³⁸

Together, they form the core structure of the AP-2 complex with two appendages connected by flexible linkers³⁹. The α and β 2 subunits form parts of the core via their N-terminal domains and the appendages via their C-terminal domains. The linker domain of β 2-adaptin is essential to interact with clathrin and the appendage domains of both α - and β 2-adaptins are involved in interactions with other adaptors and accessory proteins^{40,41} participating in the coat assembly and maturation⁴². The core of the α -adaptin subunit includes a lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) binding domain⁴³ required for binding to this lipid specifically enriched at the plasma membrane⁴⁴. This is why the AP-2 complex can only bind the plasma membrane but no other membrane-bound organelles. Both μ 2 and combination of σ 2- α subunits contribute to cargo recognition through their, respectively, tyrosine-based Yxx Φ ⁴⁵ and dileucine-based (D/E)xxxL(L/I)⁴⁶ internalization motifs (in which x is any amino acid (aa), and Φ a bulky hydrophobic aa, i.e Leu, Ile, Met or Phe).

The core of AP2 exists in two distinct conformations: a closed state, in which cargo and clathrin binding sites are hidden within the core and an open state, where these sites are accessible^{42,47}. The conformational change occurs once AP2 binds to PIP2 at the plasma membrane and is thus crucial to ensure the early stages of CME⁴⁸.

ii. Clathrin

The main component of many trafficking vesicles inside the cell is clathrin. This protein encapsulates the cargo-containing vesicle within a peculiar shape comparable to a basket (Figure 6)¹⁰. Clathrin acts as a scaffold protein in many trafficking processes between membrane-bound compartments, not only from the plasma membrane, but also from the TGN to endosomes for instance⁴⁹.



Figure 6: Schematic representations and visualization of clathrin structures

The panel (A) represents the three-legged structure of clathrin, termed triskelion. It consists of three heavy chains associated with three light chains. Clathrin heavy chains hold up the lattice structure whereas light chains have regulatory functions which influence recruitment, assembly and disassembly of the clathrin coat. (B) The clathrin self-assembly into a polyhedral cage surrounding membrane vesicle is highly regulated in cells by clathrin light chains and accessory proteins. The resulting clathrin lattice ultimately requires twelve pentagons and a variable number of hexagons to close up. (C) Electron micrograph of clathrin lattices at the cell surface. On this image, various stages of a clathrin vesicle formation can be observed.

Source : Schmid and McMahon, 2007³⁵; Heuser and Anderson, 1989⁵⁰

The basic assembly element of the clathrin lattice is the triskelion¹³ (Figure 6A). Each triskelion is composed of three 180 kDa clathrin heavy chains (CHCs) and three 25 kDa clathrin light chains (CLCs) folded in a three legs structure⁵¹. The clathrin coat as seen on CCVs is a threedimensional arrangement of triskelia in pentagons and hexagons (Figure 6B and C). Purified clathrin is able to self-assemble into cages in vitro in particular buffer conditions, but not in physiological conditions⁵². However, the addition of adaptor proteins to clathrin supports the formation of an uniform folding under physiological conditions^{16,34}. Clathrin lattice can form structures of diverse curvatures. When the coat is flat, clathrin triskelion arrangements only form hexagons. However, when the lattice is curved, like on CCVs, incorporation of pentagons is needed. Two models are under debate regarding the triskelion arrangements over time leading to the final shape of the clathrin-coated vesicle (see part II.A.2.b about invagination for details). In cells, curvature itself is generated by accessory proteins with the capacity to bend the membrane and clathrin may only stabilize this curvature⁵³.

iii. Other actors

Both clathrin and AP2 are indispensable to undergo all stages of endocytosis⁵⁴, but they need to interact with a considerable amount of partners^{55–58}. More than 50 other proteins collaborate with the two of them at specific spatial and temporal distribution during internalization (Figure 7).



Scission and movement

Figure 7: The clathrin and AP2 protein interactome

Hub-and-spoke representation of the known proteins participating to the endocytic machinery. Confirmed interactions are represented by lines and proteins are classified based on their interaction modules throughout CME. This depicts a highly regulated and complex network engaging a large amount of partners.

Source: Traub, 2011 ⁵⁹ based on Taylor, Perrais, and Merrifield, 2011 ⁶⁰

These proteins are involved in cargo selection, membrane bending, clathrin recruitment and assembly, scission or uncoating. As illustrated in Figure 8, the action of endocytic partners is highly regulated since they appear at specific locations and time during the formation of a

clathrin-coated vesicle. Some of these interacting proteins will be discussed in further details in the parts addressing their role in clathrin-mediated endocytosis.



Figure 8: Structural model of the organization of proteins in the clathrin coat

This model is based on super-resolution data indicating the specific location of endocytic partners throughout CCV formation. Six key architectural organizations are thought to occur during different steps of CME.

Source: Sochacki, Dickey, Strub, and Taraska, 2017⁶¹

b) Nucleation

Nucleation can occur spontaneously at the plasma membrane but fails to complete the endocytic cycle in the absence of cargo recognition, leading to abortive events^{62,63}. This mechanism thus prevents the folding of empty vesicles.

The exact mechanism of nucleation of early CCPs is still unclear. Different models agreed on the decisive role of plasma membrane recognition by the PIP2 binding domain of AP2. The first model proposes that two AP2 complexes already linked together by one clathrin triskelion would randomly scan the plasma membrane until binding to PIP2 ⁶⁴. But according to the alternative model, interaction between AP2 and the plasma membrane triggers the conformational change of AP2 to its open configuration, exposing the clathrin and cargo binding sites^{42,47,48}. In this model, clathrin would only be recruited after AP2 has bound the

plasma membrane. Disregarding the time-scale of clathrin recruitment, the presence of several accessory proteins during nucleation is well established.

Several endocytic pioneers are recruited at the nucleation site and assist clathrin recruitment and stabilization of AP2^{60,65} (Figure 8.a. and Table 1).

			Function	Effect on CCP
	СНС		Coat	$KD \downarrow$ initiation
ins	CLCa		Coat	KD \downarrow initiation
rote	CLCb		Coat	KD \downarrow initiation
oat p	AP2		Adaptor	KD \downarrow initiation
Major co	Dyn1		GTPase	KO no effect; A ↑ initiation and maturation
	Dyn2		GTPase	KD \downarrow initiation
		FCHo1/2	Scaffold	KD \downarrow initiation
	ins ³	Eps15	Scaffold	KD \downarrow maturation
	arriving r protei	Intersectin	Scaffold	KD \downarrow stabilization and maturation
	arly	NECAP	Adaptor	KD \downarrow maturation
	pio	CALM	Adaptor	KD \downarrow maturation
		Epsin	Adaptor	KD \downarrow Initiation

Table 1 : Keys proteins implicated in early stages of CME

Abbreviations: A, activation; AP2, adaptor protein-2; CALM, clathrin assembly lymphoid myeloid leukemia; CCP, clathrin-coated pit; CHC, clathrin heavy chain; CLC, clathrin light chain; Dyn, dynamin; Eps15, EGF-receptor phosphorylation substrate; FCHo1/2, Fer/Cip4 homology domain-only proteins 1/2; KD, knockdown; KO, knockout; NECAP, adaptin-ear-binding coat-associated protein.

Source: Mettlen, Chen, Srinivasan, Danuser, and Schmid, 2018⁶⁶

i. Scaffold proteins

Among pioneer proteins, FCHo proteins (for F-BAR domain-containing Fer/Cip4 homology domain-only proteins) are able to induce membrane curvature and thus, support the formation of the curved clathrin coat^{67,68}. The BAR (Bin, Amphiphysin, Rvs) domain

superfamily of proteins are all able to induce membrane curvature, but generate various curvature degrees. Briefly, FCHos assemble as dimers of a concave shape at the membrane surface. The binding with the PM relies on the attractive interaction between positively charged residues of the BAR domains and the negatively charged phospholipids at the membrane, hence imposing the bending⁶⁹. In the absence of FCHos, CCPs were proposed to completely disappear, AP2 and clathrin to remain cytosolic. Moreover, the same authors showed that the number of CCPs directly correlates with FCHos amounts, which demonstrate the required presence of them as nucleators⁶⁷. However, this model has been challenged by others showing that FCHo is not strictly required for CME⁶⁵.

FCHo can bind and recruit Eps15 (EGF receptor phosphorylation substrate 15) and intersectin, two other scaffold proteins, to sculpt the initial curved site. These proteins all bind to AP2 and are proposed to form together the initiation complex leading to the formation of CCPs ³⁵.

ii. Dynamin

Remarkably, dynamin, a GTPase largely studied for its role in membrane fission at later stages of CME, is also involved in the regulation of early stages of CME⁶⁶. Indeed, depletion experiments by siRNA of dynamin 2 delayed CME, intensified abortive events and super-resolution imaging indicates its presence at the beginning of vesicle formation^{61,70} (Figure 8.a.). Even if the exact mechanism underlying the contribution of dynamin during initiation remains unknown, its presence at the plasma membrane as a pioneer of CME is well-established⁶¹.

2. Maturation

The progression of the nascent CPP into productive CCP (i.e. non-abortive event) is believed to be mediated by an "endocytic checkpoint/ restriction point" determined by the coat composition and cargo loading^{63,66,71}.

a) Cargo selection

A wide variety of cargoes are carried through the clathrin-mediated endocytosis pathway. Some receptors are constitutively internalized, whether or not the ligand is bound, such as transferrin receptors (TFRs) and low-density lipoprotein receptors (LDLRs)^{72,73}. The internalization of numerous other receptors depends on ligand-receptor interaction which "activates" the receptors, allowing their recognition by specific endocytic adaptors. Cargo recruitment relies on the recognition of diverse sorting signals located at the cytosolic tail of transmembrane receptors by clathrin coat constituents^{37,74}. Endocytic signals diversity allows a high flexibility of partners and regulators and prevents competition among proteins for internalization (Table 2).

Signal	Examples of cargo	Recognition protein or domain	Architecture	Ref
ҮххØ	Transferrin receptor, Dishevelled 2	μ2 subunit of AP2	α-trunk α-appendage Individual μ σ	46, 76
[DE]xxxL[LI]	CD4, CD3γ, tyrosinase	α - σ 2 subunits combination of AP2	AP2	
[FY]xNPx[YF]	LDL receptor, Megalin, Notch	PTB domain of ARH, Dab2 and Numb	ARH Dab2 Numb	75
Phosphate group	GPCRs	β -arrestin 1 and 2	Arrestin β-arrestins	77
Ubiquitin	EGF receptor, MHC class I	UIM domains of Eps15 and epsins	Eps15	78,79

 Table 2 : Sorting signals recognized by the endocytic machinery

Abbreviations: Ø indicates a bulky hydrophobic amino acid (Leu, Met, Ile, Phe or Val) and X is any amino acid. ARH, autosomal recessive hypercholesterolemia; Dab2, disabled 2; EGF, epidermal growth factor; EPS15, EGFR substrate 15; GPCR, G protein-coupled receptor; LDL, low-density lipoprotein; MHC, major histocompatibility complex; PTB, phosphotyrosin-binding; UIM, ubiquitin-interacting motif.

Source: adapted from Traub, 2009³⁷; McMahon and Boucrot, 2011⁵⁸

The YxxØ and [DE]xxxL[LI] motifs are the most common sorting signals, both recognized by the AP2 complex, hence making AP2 a key regulator of cargo recognition^{74,75}. After the binding to the PM during nucleation, AP2 switches to its open conformation, enabling cargo accessibility and, with the assistance of clathrin, enhancing its affinity for it^{79–81}.

However, additional adaptors appear to be involved in cargo selection. Evidences of alternative adaptors for CME were provided by experiments performed on AP2 depleted cells. In the absence of AP2, these cells are still able to internalize LDLR and EGFR through CME, while internalization of TFR is impaired^{82,83}. The efficiency of these other adaptors is mediated by four features: an ability to bind cargo, PIP2 at the PM, clathrin and AP2. All of phosphotyrosine binding (PTB) domain containing proteins, β -arrestins and epsins display the above-mentioned characteristics⁷⁴.

Regarding the [FY]xNPx[YF] motif, it was firstly found in members of the LDL receptor family and several reports demonstrated robust interactions with the disabled homolog 2 (Dab2) and the autosomal recessive hypercholesterolemia (ARH) proteins. This sequence is specifically recognized by the phosphotyrosine-binding domain (PTB) of these proteins, although their binding preference is given to non-phosphorylated tyrosine. Although, since fibroblasts derived from patients with ARH disorder have normal LDLR activity, proteins with PTB domains might have a redundant function⁸⁴. Another adaptor takes part in this family: Numb. These three members of PTB domain protein are all able to bind AP2 through its ear domain of the α subunit, and has the ability to bind and to biologically antagonize the receptor Notch. Numb acts as a cell fate determinant by asymmetrically partitioning during mitosis.

Other adaptors include β -arrestins that are the specific adaptors for G protein-coupled receptors (GPCRs) and epsins, especially their ubiquitin interacting motifs (UIMs).

The clustering of cargo molecules to CCPs appears to be decisive for maturation. Indeed, overexpression of transmembrane receptors at the plasma membrane increases the amount of productive CPPs without altering their lifetime⁷². Similarly, live-cell imaging of EGFR and some GPCRs (G protein-coupled receptors) showed an aggregation of these receptors upon ligand binding followed by a recruitment to preformed endocytic sites^{85,86}. In addition, experimental clustering of receptors at the cell surface leads to local CCPs nucleation/maturation⁷². Thus, it is nowadays well accepted that cargos play an important role in CCPs maturation.

b) Invagination

Generation of membrane curvature necessitates a local asymmetry within the two bilayers of the plasma membrane⁸⁷. Membrane bending can be due to at least four different means^{53,58} (Figure 9). ⁵⁹



Figure 9: Four different mechanisms causing membrane deformation

Schematic representation of the means which can be used by proteins to induce local membrane bending. Basically, the asymmetry between the two leaflets is responsible for curvature. (A) Lipids with large head groups can locally aggregate at a one leaflet membrane. (B) Proteins containing amphipathic helix can insert them into one layer of the membrane. (C) Previously curved proteins can bind to the membrane and thus coerce its bending. (D) Finally, local clustering of proteins associated to membrane can cause deformation.

Source : Merrifield and Kaksonen, 2014⁵⁹

First, membrane composition in lipids, especially the ones with large head groups, can induce membrane curvature by clustering (Figure 9.A)⁸⁸. Secondly, insertion of amphipathic helices of several proteins into the membrane bilayer leads to membrane invagination⁵³. This is the case for epsins, adaptors proteins recruited by AP2 and Eps15 right after nucleation (Figure 9.B.)⁶¹, whose helix (ENTH domain) penetrates the external leaflet of the plasma membrane, thus increasing the area of this leaflet while the external one remains unaffected ⁸⁹. The difference of local area between the two leaflets forces the membrane to bend. Epsin in turn boosts clathrin recruitment and its assembly to stabilize the curvature. Thirdly, association between the PM and already curved proteins such as BAR domain family proteins is able to locally deform the membrane (Figure 9.C)⁹⁰. For instance, the early arrival protein FCH0 is part of this family, but also the late arrival amphiphysin and sorting nexin-9 (SNX9). Finally, even transmembrane

proteins crowding at a specific location can promote membrane bending (Figure 9.D)⁹¹. During clathrin-mediated endocytosis, these mechanisms can collaborate for bending membranes and propagation into CCPs. While clathrin is able to deform membranes in vitro, it is generally accepted that in vivo, the clathrin coat only stabilizes the curvature generated by the other factors described above.

The relation between clathrin polymerization and membrane invagination remains obscure. A first model where clathrin would polymerize directly as a curved structure to form the coat face a second one in which clathrin first assembles as flat lattices made of hexagons and then reorganize into pentagons and hexagons^{92,93}. The debate about these two models is still very active in the field although late works favor the second model^{94–96}. The elegant work of Avinoam et al., consistent with the flat-to-curved model, indicates the maintenance of a constant coated membrane area while the curvature increases during invagination. After performing fluorescence recovery after photobleaching (FRAP), they observed a rapid turnover of clathrin at all stages of CME, giving even more credit to the second theory which fits to their experimental data⁹⁴.

c) Regulators of vesicle size

Clathrin-coated vesicles display a remarkably size homogeneity, indicating a fine-tune regulation of their diameters. Clathrin assembly lymphoid myeloid leukemia (CALM) and NECAP (or adaptin ear binding coat associated protein) proteins are two regulators involved in diameter adjustment of CCVs^{97,98}. Regarding NECAP, its function in CCVs size regulation is mediated by a Pleckstrin Homology (PH)-like domain which sustains the closed conformation of AP2, thus limiting the binding of accessory proteins, including CALM and its neuronal homologue AP180. CALM presents an AP180 N-terminal homology (ANTH) domain similar to epsin N-terminal homology (ENTH) domain of epsins, able to bind PIP2 but lacking the amphipathic helix involved in membrane bending. CALM depletion raises the amount of closed CCVs (vesicles which are about to bud) compared to open ones, and doubles CCVs diameter as well. In this respect, both NECAP, even indirectly, and CALM are master regulators of CCVs size and cargo uptake capacity.

3. Scission

a) BAR proteins

During maturation, as the membrane curvature continuously increases and the membrane neck turns thinner, the binding with FCHo proteins and Eps15 becomes unfavorable and they progressively dissociate from the neck. The high degree of curvature at late stages of CME benefits more curved proteins such as different subfamilies of BAR proteins. Indeed, the X-BAR and N-BAR proteins SNX9, amphiphysin and endophilin get engaged at the neck of the CCPs only at the late stage of their maturation^{61,99}. They mediate the constriction of the neck through their BAR domain, induce local actin polymerization by recruiting N- WASP and concentrate dynamin at the neck (Figure 10)^{100,101}.



Figure 10: Role of BAR proteins in membrane shaping and scission

Schematic representation of the participation Bin/Amphiphysin/Rvs (BAR) proteins during CME. F-BAR proteins displaying a low curvature including formin-binding protein 17 (FBP17) and sorting nexin 9 and 18 (SNX9/18) are firstly recruited to nascent clathrin-coated pits. However, at late stages of CME, highly curved N-BAR proteins such as endophilin and amphiphysin replace F-BAR proteins, and localized at the neck of deeply invaginated CCPs to promote dynamin recruitment and scission.

Source: Kaksonen and Roux, 2018 55

b) Dynamin

Scission itself, when the vesicle physically detach from the PM, is mediated by the large GTPase dynamin which forms a collar around the membrane neck and constricts it until it breaks. Dynamin was discovered in 1989 and rapidly designated as the core of the endocytic fission process ^{102,103}. Following this discovery, lots of studies had focused on dynamin to decipher the underlying machinery beneath membrane scission¹⁰⁴.

Dynamin has three isoforms: two of them (1 and 3) are expressed in neurons, and dynamin 2 which is ubiquitous. This protein has the particularity to self-assemble into helices, wrapping themselves around membrane tubes. Through the hydrolysis of GTP and a conformational change, the collar tightens around the tubular-shaped membranes and breaks them. Interfering with dynamin function causes impairment of CME, as exemplified by a strong reduction of transferrin uptake and by an increase in the number of deeply invaginated CCPs at the plasma membrane¹⁰⁵⁻¹⁰⁷.

Even though dynamin is already recruited at earlier stages of CME, the major burst of dynamin recruitment occurs during scission at the neck of endocytic vesicles, implying a de novo recruitment by different partners. These partners were identified as the BAR proteins amphiphysin, endophilin and SNX9^{108–110}. All together, they cooperate to enhance dynamin assembly and its GTPase activity, thus supporting vesicle detachment.

Another significant partner of dynamin is actin. This protein polymerizes at the membrane neck at about the same time as dynamin, just prior to scission and several studies demonstrated a closed interplay between actin and dynamin which plays a crucial role in vesicle fission.

c) Actin

The energy required to deform a membrane depends on membrane elasticity. Even if clathrin and accessory proteins can bend membranes locally, they sometimes requisite the assistance of additional forces driven by the actin cytoskeleton in order to counteract membrane tension^{111,112}. Actin polymerization occurs transiently and at late stages of CME in mammalian

cells^{113,114}. The key proteins in cytoskeleton recruitment are WASP family¹¹⁵. These proteins are enrolled at the membrane neck by SNX9, endophilin and syndapin and then mediate actin polymerization through the Arp2/3 complex^{116–118}. Actin is localized at the edge of deeplyinvaginated CCPs and reaches a maximum recruitment at about the same time as dynamin recruitment^{119,120}. The assembly of actin filaments at the rim of CCPs would generate an actin flow from the plasma membrane toward the interior of the cell, and thus propel the vesicle away from the membrane¹²¹. The closed relationship between dynamin and actin, together with BAR proteins, was shown to be necessary for dynamin function in fission. By the use of latrunculin B in cells expressing WT dynamin or a non-functional mutant of dynamin, a powerful and mandatory cooperation between dynamin and the actin network was demonstrated for vesicle separation(Figure 11)^{122,123}



Figure 11: The role of dynamin during CME

(a) Representation and visualization of dynamin and its effects on CME. The absence of dynamin induces an impairment of vesicle scission, as compared to regular CME (top right panel), while actin (in green) keeps polymerizing, leading to the formation of a tubular shape at the neck of the CCP (bottom right panel).

(b) Sequence of endocytic proteins recruitment throughout CME. Time zero corresponds to membrane fission. A burst of recruitment of dynamin, actin and the membrane bending protein endophilin is observed just prior scission, suggesting a crucial role of these proteins in vesicle detachment.

Source : Ferguson and De Camilli, 2012¹²⁴

4. Uncoating

Clathrin-mediated endocytosis ends with the disassembly of the coat right after vesicle scission to enable the fusion of the endocytic naked vesicle with early endosomes. The dismantlement of the clathrin lattice back to soluble triskelia is undertaken by the ATPase heat shock cognate 70 (Hsc70) and auxilin¹²⁵⁻¹²⁷. Regarding the occurrence of uncoating at this precise time, the question can be justify by a defect in the clathrin cage left during vesicle scission which is believed to be the starting location of disassembly⁵⁷.

Auxilins encompass two homologues : auxilin 1, expressed in neurons and auxilin 2, also called GAK for cyclin G-associated kinase which is ubiquitously expressed. This proteins recruitment happens shortly after dynamin-mediated fission and precedes clathrin disappearance related to uncoating^{60,128,129}. Auxilins harbor a clathrin binding domain and a DnaJ domain interacting with Hsc70^{130,131}. Auxilin is recruited to the extremity of a triskelion, which colocalizes with the center of a neighboring triskelion within the lattice¹³². Once positioned there, auxilin recruits up to three Hsc70 proteins, hence initiating the coat disassembly (Figure 12)^{133,134}.



Figure 12 : Mechanism of uncoating by auxilin and Hsc70

First, an auxilin binds to the clathrin coat (a) and recruits one Hsc70 linked to ATP (b). ATP is hydrolysed in ADP by Hsc70 and this is the signal for a second recruitment of Hsc70:ATP to the coat by auxilin (c). The reaction is repeated once again (d, e). Each time the ATP hydrolysis occurs, the affinity of Hsc70 for clathrin increases until exceeding the one between clathrin and auxilin, and this allows coat disassembly (f).

Source: website How are clathrin-coated vesicles uncoated, 2018¹³⁵
These three molecules of Hsc70 are first bound to ATP, and arrive sequentially at auxilin location. ATP hydrolysis by Hsc70 is stimulated through the contact with both the DnaJ domain of auxilin and clathrin. This induces local distortion of the clathrin lattice by a conformational change which clamps firmly onto clathrin heavy chain and locks this configuration until the arrival of another Hsc70. Three molecules of Hsc70 are required for maximal stimulation of Hsc70 activity and maximal binding of clathrin. The distortion generated at this stage is enough to disrupt the clathrin coat¹³⁶.

B. Vesicle sorting

Once clathrin-coated vesicles got rid of their coat, naked vesicles rapidly fuse and deliver their cargoes to a common sorting compartment in the cells, called early endosomes (EEs)¹³⁷. EE are highly dynamic organelles derived from the fusion of endocytic vesicles of several pathways¹³⁸. The peculiar characteristic of early endosomes is to be subdivided into tubular membranes dedicated to recycling and large vesicles containing membrane invaginations (therefore described as multi-vesicular) further involved in the degradative pathway (Figure 13).¹³⁹

The mildly acidic environment (pH ~ 6,5) within EEs induces, for most cargoes, the dissociation between receptors and ligands¹⁴⁰. Receptors are generally recycled at the plasma membrane while ligands are routed to late endosomes and lysosomes for degradation¹⁴¹. Not all cargoes share the same fate, many of them are targeted to different intracellular destinations¹⁴². Transferrin, for example, is internalized with its receptor and both are recycled whereas EGF/EGFR are targeted together to lysosomes for degradation^{143,144}. The different paths taken by endocytic molecules are further detailed below.



Figure 13: Endosome sorting pathways

After vesicle internalization, cargoes are delivered to early endosomes (EEs). Within minutes, EEs support receptor recycling to the PM, directly of via recycling endosomes. Then, endosomes can mature from the multivesicular domain of EEs into late endosomes (LEs). During maturation, endosomes move toward the perinuclear space through microtubules. Simultaneously, newly synthetized lysosomal hydrolases are delivered to nascent LEs to acidify it to prepare for the encounter with lysosomes. LEs fuse with lysosomes and first form a transient organelle, the endolysosome ensued by conversion into lysosome.

Source : Huotari and Helenius, 2011¹³⁹

1. Recycling

Receptors can be recycled by two different means upon the delivery of cargoes to early endosomes. The endosome develops narrow-diameter tubules at the site where endocytic vesicles fuse with EEs. As a consequence of the organelle acidity, ligand-bound receptors dissociate from each other. The restricted volume within the thin tubules enables mostly receptors bound to membranes to be located in this area, whereas the now-soluble ligands converge at the center of the EEs. Vesicles generated from these tubules thus contain a small amount of lumen and a large fraction of membrane along with receptors^{137,145}. These vesicles are then routed either directly to the plasma membrane for fast recycling ($t_{1/2} = 5 \text{ mn}$) or they transit through a recycling endosome compartment (ERC) where they undergo slow recycling ($t_{1/2} = 15-30 \text{ mn}$)^{146,147}. Fast recycling is regulated by the small GTP-binding protein Ras-associated

binding 4 (Rab4) which addresses the newly-formed vesicles to the PM, whereas slow recycling is mediated by both Rab4 and Rab11 at recycling endosomes¹⁴⁸. Receptors are mostly sorted for recycling unless they present specific targeting informations for lysosomal degradation.

The early endosome fuses with incoming vesicles for about 5-10 minutes and then undergoes several tubules budding until up to 80% of its surface is recycled back to the plasma membrane (PM). This "geometry-based sorting" provides an efficient way to separate molecules fated to recycling from degradation without requirement of any active transportation of soluble content. After budding, the remaining part of early endosomes starts to mature and acquires the properties of late endosomes.

2. Degradation

The first step in targeting transmembrane proteins to degradation via the endosome/lysosome pathway is usually ubiquitination. Ubiquitin is a protein that can be covalently linked to lysine residues at the cytoplasmic domain of transmembrane proteins. It was first described for its role in protein degradation through the proteasome by the formation of poly-ubiquitin chains. However, mono-ubiquitination signals lead to sort proteins for degradation by lysosomes¹⁴⁹. Recognition of these signals relies on the hepatocyte growth-factor regulated tyrosine-kinase substrate (Hrs) and its ubiquitin interacting motif (UIM). Hrs can bind to clathrin lattices located at the limiting membrane of endosomes involved in degradation¹⁵⁰. This results in the formation of microdomains which cluster cargoes aimed for degradation.

The maturation from early endosomes to late endosomes starts once recycling of proteins and lipids unlabeled for degradation is achieved. Everything that have not been recycled, including lipids and soluble proteins, remains instead in the lumen or at the limiting membrane of EEs while they evolve in late endosomes. Proteins destined to degradation get encapsulated into intraluminal vesicles (ILVs) which bud from the limiting membrane of endosomes within their own lumen. These ILVs give their characteristic appearance to multivesicular bodies (MVBs). Meanwhile, MVBs translocate to the center of the cell through an association with microtubules

until they reach lysosomes. In the end, fusion of MVBs with lysosomes containing proteolytic enzymes leads to the dissolution of proteins^{139,151}.

C. Physiological functions

The internalization of cargoes from the plasma membrane through clathrin-mediated endocytosis is involved in many key cellular functions. Indeed, the distribution of receptors at the PM considerably affects the ability of the cell to sense and respond to the ever changing conditions of its environment. CME regulates the cellular uptake of cargoes implicated in development, growth control, cell differentiation, neurotransmission, polarity and is even exploited by viruses, toxins and bacteria to force their own entry into cells. Some of these functions will be discussed in this section.

1. Receptors internalization

The first known characteristic of CME is the selective internalization of receptors. However, depending on the receptor, this uptake can be constitutive or stimulated¹⁵²⁻¹⁵⁴.

Constitutive internalization refers to receptors continuously internalized with or without ligand, and are usually recycled at the plasma membrane within minutes. Receptors taken up constitutively include non-signaling receptors involved in the uptake of nutrients. The classical example of such constitutive internalization is the transferrin receptor (TfR) which is able to bind transferrin loaded with iron but can be uptaken also in the absence of its ligand¹⁵⁵. Tf trafficking pathway via CME has been extensively studied and Tf is thus used as a quantitative tool to measure levels of endocytosis^{156,157}. The low-density lipoprotein receptor (LDLR) is also a well-established receptor to undergo constitutive endocytosis^{82,158,159}.

Stimulated or ligand-induced internalization, is reported to be the main pathway exploited by receptor tyrosine kinases (RTKs) such as EGFR, and G protein-coupled receptors (GPCRs) including β 2 adrenergic receptor^{160,161}. For these receptors, ligand binding induces dimerization

(in the case of RTKs) or a conformational switch (for GPCRs) both essential for their recruitment into CCPs. Following their uptake, receptors are either recycled back to the plasma membrane or degraded along with their ligands^{162,163}. In these cases, the role of CME is to remove active signaling receptors from the plasma membrane and thus terminate signaling events by routing them for degradation.

Internalization of receptors at the plasma membrane through clathrin-mediated endocytosis allows a high flexibility in cellular responses to the microenvironment by finely controlling the quantity and distribution of receptors at the cell surface and thus the uptake of nutrients, growth factors and hormones.

2. Signaling

It was long believed clathrin-mediated endocytosis simply regulates cell signaling by influencing the availability of receptors accessible to the ligand at the plasma membrane. However, emerging evidences indicate a role for clathrin-coated structures as signaling platforms.

a) Signal termination

CME primary function is to terminate the signal through the removal of activated receptors from the cell surface (Figure 14). Signal extinction can occur either via the degradation of receptors inside lysosomes or just by separating receptors from plasma membrane substrates such as PIP₂ or membrane-bound mediators^{163,164}.



Figure 14: Down-regulation of signaling mediated by CME

Schematic representation of activated receptors and their signalization followed by signal termination through clathrin-mediated endocytosis. (1) Ligand-induced activation of receptor tyrosine kinases (RTKs) or G proteincoupled receptors (GPCRs) at the plasma membrane. Activated receptors initiate signaling at the cell surface via autophosphorylation for RTKs or when the G α subunit is bound to GPCRs. (2) Then, these receptors are recruited to clathrin-coated pits and delivered to endosomes after uptake (3). The separation of receptors from their membrane-associated mediators is the first step of signal attenuation. Some receptors are routed for degradation in lysosomes thus leading to signal termination through proteolysis

Source : Sorkin and von Zastrow, 2009¹⁶⁵

b) Signal propagation

However, the internalization of ligand-bound receptors into endosomes, which also function as signaling platforms, can promote sustained signaling instead of ending it^{166,167}. Indeed, several endosomal properties such as long residence time of active receptors in endosomes, specific resident proteins involved in signaling complex formation, acidic pH which favors the activity of proteolytic enzymes, can support long-lasting signaling¹⁶⁸. Moreover, ligand concentration seems to be important to target receptors to the degradative or the recycling pathways. For example, low concentrations of EGF assign EGFR to internalization for recycling and then sustained signaling at the PM, while receptor saturation under high concentration of ligands targets the receptors for degradation¹⁶⁰. This example clearly illustrates the reciprocal relationship between cell signaling and the endocytic pathway.

c) Signaling platforms

As previously mentioned, clathrin-coated structures can also act as signaling platforms. In some cell types, such as HeLa, hepatocytes or osteoblasts among others, in addition to the dynamic regular clathrin-coated pits, flat clathrin lattices can be observed (Figure 15).



Figure 15: Flat clathrin lattices at the cell surface

Electron micrograph of the cytoplasmic face of the plasma membrane. Clathrin-coated pits can be identified as roundish vesicles with a basket-like shape where clathrin assembles in hexagons and pentagons while large flat clathrin lattices consist mainly of hexagons.

Source: Hirst and Robinson, 1998¹⁶⁹

For a long time, flat clathrin lattices, also termed plaques, were not taken in account while studying endocytosis. Indeed, and unlike clathrin-coated pits, plaques are long-lived structures, from minutes to hours, at the plasma membrane and thus are not believed to perform endocytosis^{170,171}. Even if the formation of clathrin-coated vesicle seems to build first as flat clathrin assembly at the plasma membrane, the area of clathrin-coated plaques, as seen in Figure 15, is much more important than the one of CCPs. However, an increasing amount of studies started to focus on the role of these clathrin-coated plaques and their role in signaling^{172,173}. Long-lived structures can appear under different conditions, for instance high membrane

tension^{112,174,175}. A recent work has also determined a correlation between these structures and substrate rigidity¹⁷⁶. These plaques would retain receptors at the cell surface, and hence promote signaling instead of terminate the signal after internalization. Substrate rigidity enhances adhesion and integrins at clathrin-coated structures prevent budding. This phenomenon is referred as "frustrated endocytosis". Further experiments have linked frustrated endocytosis with sustained signaling, especially the Erk pathway activated by the EGFR at the plasma membrane¹⁷⁶. Accordingly to these results, clathrin-coated plaques would act as signaling platforms by being a physical support of receptors at the cell surface.

d) Migration

CME can also spatially restrict signaling responses to guidance cues. For instance, this is the case for migrating border cells in Drosophila melanogaster¹⁷⁷. RTK signaling is limited to the leading edge of border cells under the control of two early stages regulators of RTKs endocytosis (Cbl and Sprint), thus providing a RTK-directed migration. This emphasizes the necessity of CME for localized signaling which preserves the spatial information of ligand gradients and hence their use for directional migration. Another illustration of the involvement of clathrinmediated endocytosis in migration is its role in integrins trafficking^{178,179}. Many processes such as cell adhesion, migration and polarity maintenance depend on the specific location of integrins over the cell surface. Integrins are the major adhesion receptors of the cell to the extracellular matrix (ECM). Their clustering results in the formation of adhesive structures called focal adhesions (FAs) that enable a connection between the ECM and the actin cytoskeleton. FAs are used as traction sites during mesenchymal cell migration where the cell forms protrusions and attaches to the ECM at the leading edge followed by the propulsion of the cell body forward. The formation and disassembly of integrins during this process in mediated by clathrin-mediated endocytosis through their adaptors ARH and Dab2180. In addition to FAs and by similarly clustering integrins linked to the ECM, clathrin-coated structures themselves have also been reported to behave as adhesive structures, hence supporting migration in collaboration with focal adhesions¹⁸¹.

3. Specialized endocytic processes

a) Polarity maintenance

In polarized epithelial cells, the plasma membrane is divided in two biochemically and functionally distinct areas: the apical and basolateral domains. The two PM leaflets have different lipids and protein composition and the separation between them is maintained via tight junctions. These tight junctions prevent membrane components to diffuse across the whole membrane, and this gives the cell its polarity. In order to preserve this polarity, specialized trafficking mechanisms are required^{182,183}. The first one involves the polarized delivery of newly synthesized proteins sorted by the TGN. The second one implicates recycling after internalization and this one involves CME specifically. In this case, proteins can either go back to their membrane domain of origin or they can be delivered at the opposite side of the cell in a process called transcytosis¹⁸⁴. For instance, in rat hepatocytes, all the membrane proteins sorted by the TGN are routed to the basolateral membrane and the part of them destined to the apical membrane requires transcytosis to be redirected to the apical pole¹⁸⁵. CME thus provides the mean for the proper relocation of apical and basolateral proteins which requires transcytosis to reach the correct membrane domain. It is also a way to rectify mislocalization due to sorting defects¹⁸⁶. Moreover, the role of CME in polarity is also decisive in regulation of the appropriate levels of key polarity proteins.

a) Neurotransmission

Another important specialized function of CME is its role in neurotransmission^{187,188}. Communication among neurons occurs through the release of chemicals signals, the neurotransmitters, to surrounding neurons (Figure 16).



Figure 16: Function of CME in synaptic vesicle recycling

During signal transmission at synapses, neurotransmitters are released into the synaptic cleft and captured by the postsynaptic neuron. Meanwhile, synaptic components are sorted into clathrin-coated pits through AP2 and accessory proteins. Synaptic vesicles are then recycled and translocated to endosomes where they are reloaded with neurotransmitters for another round of release.

Source : McMahon and Boucrot, 2011 57

Neurotransmitters are delivered by synaptic vesicles in the synaptic cleft and captured by the adjacent neuron. The now-empty synaptic vesicle is then recycled through CME and turned into a new competent synaptic vesicle able to acquire a new loading of neurotransmitters. Highly active pre-synaptic neurons can hold thousands of vesicles and this necessitates a very efficient and local recycling of synaptic vesicle components to undergo successive rounds of neurotransmission. CME in neurons is not only important for synaptic vesicle recycling, but as well to regulate the size and protein composition of these vesicles¹⁸⁹. Moreover, specificity of CME in neurons is underlined by the presence of brain isoforms of adaptor proteins such as AP180, epsin 1, amphiphysin 1 and dynamin 1 and a high concentration of CME components compared to other tissues.

D. Endocytosis and disease

Considering the major role of clathrin-mediated endocytosis in a wide variety of physiological processes, dysregulations of this membrane trafficking pathway are often associated with diseases¹⁹⁰ (Table 3).

The first identified association between a disease and a defect in clathrin-mediated endocytosis was in autosomal recessive hypercholesterolemia (ARH). Patients affected by this disorder display elevated plasma LDL levels caused by a defect in internalization and thus degradation, even if LDL receptors were operationals. This deficiency has been shown to be linked to mutations in the phosphotyrosine binding (PTB) domain of the adaptor protein ARH⁸⁴.

CME alterations are also found in diverse neuronal disorders, including schizophrenia, bipolar disorder, the neuromuscular disorder centronuclear myopathy, the Charcot-Marie Tooth disease or even Alzheimer. These changes include mutations, single nucleotide polymorphisms, or altered expression of proteins implicated in CME such as amphiphysin, dynamins, endophilin, CALM or synatopjanin. However, it is worth noting that even if these modifications have been reported in these diseases, the causal relationship between them and the occurrence of the disorders remains to be elucidated. Another point to take into consideration is that the alterations mentioned affect only accessory proteins, the core components of clathrin-mediated endocytosis remain unchanged. Also, due to the high resilience of CME thanks to its multiple adaptors, mutations probably don't impair endocytosis itself¹⁹¹. However, regulated cargo recruitment appears to be defective, leading to the aggregation of proteins responsible of several diseases.

As discussed previously, endocytosis is an important regulator of signal transduction, cell polarity, cell migration, in addition to its common role in nutrients uptake. Given its crucial role, aberrant endocytic machinery or unbalanced CME can lead to uncontrolled cell proliferation, epithelial-mesenchymal transition (EMT), apoptosis escape and cell invasion, all of them being hallmarks of cancer¹⁹². Indeed, the transformation of healthy cells into cancer cells involves the cooperation of multiple oncogenic mechanisms in which CME plays a

synergistic role (Figure 17). For instance, the loss of the apical-basal polarity, the sustained signaling due to an enhanced receptors recycling, or the accelerated turnover of integrins all rely on clathrin-mediated endocytosis.



Figure 17: Contribution of derailed endocytosis in malignant transformation

CME is involved in all steps that take part of cancer progression. This includes polarity disruption with redistribution of major polarizing actors and hence epithelio-mesenchymal transition (EMT). Altered sorting of receptors via ubiquitination or sustained signaling promotes cell proliferation. Unusual integrins trafficking also contribute to another malignant marker, cell migration.

Source: Mosesson, Mills, & Yarden, 2008 190

Moreover, many alterations of proteins implicated in the endocytic pathway are found in tumors (Table 3). A high proportion of alterations are observed in blood cancer leukemias and lymphomas, mostly fusion genes or overexpression of clathrin heavy chain, Eps15, CALM, endophilin, HIP1 and HIP1R. By contrast, alterations encountered in solid tumors encompass essentially downregulations and somatic mutations of CHC, Eps15, Dab2, HIP1, βarrestin1 and Numb. This implies the high resistance of differentiated cells (blood cells) to substantial perturbations compared to highly dividing cells (solid tumors) which are more sensitive and display only moderate alterations.

Disease category	Protein	Gene	Disease	Alterations
Metabolic syndrome	ARH	LDLRAP1	Autosomal recessive	Mutations
2			hypercholesterolaemia	
Neuronal disorder	Amphiphysin	BIN1	Schizophrenia	Increased expression
	Endophilin	SH3GL1	Schizophrenia	Decreased expression
	Dynamin	DNM1	Schizophrenia	Increased expression
	Synaptojanin	SYNJ1	Bipolar disorder	Mutations
	Amphiphysin	BIN1	Autosomal dominant	Mutations
			centronuclear	
			myopathy	
	Dynamin	DNM2	Autosomal dominant	Mutations
	24		centronuclear	
			myopathy	
			Dominant intermediate	
			Charcot-Marie-Tooth	
			disease	
	CALM	PICALM	Alzheimer	SNPs
	Amphiphysin	BIN1	Alzheimer	SNPs
Cancer	Clathrin Heavy Chain	CLTC	Inflammatory	CLTC-ALK fusion
			Myoblastic Tumour	CLTC-ALK fusion
			Large B-cell lymphoma	CLTC-TFE3 fusion
			Paedriatic renal	Somatic mutations
			carcinoma	
			Renal and Breast	
			cancers	
	Eps15	EPS15	Acute Myeloid	EPS15-MLL fusion,
			Leukaemia	Somatic mutations
			Lung cancer	Somatic mutations
	CALM	PICALM	Acute Myeloid	PICALM-AF10, -MLL
			Leukaemia	fusions
			Acute Lymphoblastic	Somatic mutations
			Leukaemia	
	Dab2	DAB2	Ovarian, Prostate,	Downregulated
			Bladder, Breast	
			Oesophageal cancers	
			Colorectal carcinoma	
	Endophilin	SH3GL1	Acute Myeloid	EEN-MLL fusion
			Leukaemia	Somatic mutations
	HIP1	HIP1	Chronic	HIP1-PDGFRB fusion,
			Myelomonocytic	Somatic mutations
			Leukaemia	Somatic mutations
			Lung and Breast cancer	
	HIP1R	HIP1R	Colon cancer	Overexpressed
			Chronic Lymphocytic	
			Leukaemia	
	Cortactin	CTTN	Primary breast	Overexpressed,
			carcinoma	amplification
	βArrestin1	ARRB1	Breast cancer	Somatic mutations
	Numb	NUMB	Breast cancer	Downregulated

Table 3 : Dysregulation of clathrin-mediated endocytosis in diseases

Abbreviations: ARH: autosomal recessive hypercholesterolemia; CALM: Clathrin assembly lymphoid myeloid leukemia; SNP : single-nucleotide polymorphism; HIP : Huntingtin Interacting Protein 1; HIP1R: Huntingtin Interacting Protein 1 Related

Source: adapted from McMahon and Boucrot, 2011 57,193

On top of that, cancer progression is tightly associated with changes of the microenvironment which forms a niche with distinctive biochemical and physical properties. Indeed, exposure to growth factors, contacts with adjacent cells or mechanical changes of the ECM make the response mediated by clathrin-mediated endocytosis crucial to cancer evolution^{176,194,195}. These matters will be discussed in greater details in parts II and III below.

III. The tumor microenvironment

Normal tissue homeostasis is preserved by a dynamic cross-talk between cells and their microenvironment. The microenvironment itself consists of the insoluble extracellular matrix (ECM); the stroma containing fibroblasts, adipocytes, vascular and immune cells; and extracellular molecules such as cytokines and growth factors. The ECM is the structural support network consisting in fibrous proteins like collagen, elastin, fibronectin and laminin and the space between them is filled by viscoelastic gels of polysaccharides. ECM network is extremely dynamic, its composition and organization fluctuate over time, especially under the control of matrix metalloproteinases (MMPs and ADAMs)¹⁹⁶. During cancer development, several changes occur within the microenvironment which co-evolve with tumor cells. The extracellular matrix is usually deregulated in cancer and this anomalous ECM also disturbs the behavior of stromal cells, leading to the formation of a niche supportive to cancer cells. Such a favorable field promotes cell survival, sustained proliferation, angiogenesis and malignant invasion, thereby driving tumor progression¹⁹⁷. Extensive studies have been conducted on the interplay between tumor and its microenvironment and are discussed in multiple reviews^{198–200}.

A. Mechanical changes

Notably, the ECM governs many of the cellular responses characterizing the cancer hallmarks²⁰¹. This suggests that biochemical and biophysical cues of the ECM are exploited by tumor cells for progression. Indeed, many malignantly transformed tumor cells have acquired the capacity to secrete their own extracellular matrix proteins and to alter the expression of remodeling proteins, allowing them to survive in hostile environments²⁰². As the tumor grows and cancer cells proliferate in a restricted space, they are subjected to many physical forces which, with the coordinated action of stromal cells, promote invasion (Figure 18).



Figure 18: Tumor growing and physical forces

Breast cancer example of tumor development. First, healthy cells undergo transformations into cancer cells. These alterations promotes proliferation and enhanced survival which disrupt normal mammary gland organization. Cancer cells keep growing, in parallel to stroma modifications leading to an increased stiffness. The expanding tumor mass starts to push on the surrounding environment, while the tissue exerts an opposite force to counteract this pressure. The accumulation of all this forces on the tumor favors tumor migration and invasion.

Source: adapted from Butcher, Alliston, and Weaver, 2009²⁰³

Matrix stiffening

The tumor microenvironment is commonly stiffer than normal tissues and this mechanical change correlates with a high metastatic potential and a poor prognosis¹⁹⁹. Considering this, tumor detection is first based on palpation of the tissue, when accessible, and can be reinforced by magnetic resonance imaging or ultrasounds^{204,205}. These techniques are able to measure the elasticity (*E*, also called Young's modulus) of a tissue. It corresponds to the ratio of the force applied on an object per unit area (N/m² or Pascals (Pa)), i.e. the stress, to the deformability of this object, i.e. its strain. Since strain is dimensionless, elasticity is expressed in Pascal (Pa). However, it is important to make the distinction between stiffness which refers to tissue deformability, and solid stress defined by the force per unit area exerted by the solid

components of a tissue^{206,207}. Consequently, stiffer tissues do not always display a high Young's modulus and high elastic energy does not necessary mean a stiffer tissue. In solid tumors, the stiffness is mostly determined by the ECM composition and organization, whereas solid stress comes from all the physical forces involved in tumor growth.

Measurements of tissue stiffness point out a considerable gap among tissues, from 17 Pa (fat) to 89 kPa (spinal cord) in humans and up to 310 MPa for the Achille's tendon in rats (see also Figure 19 for representation)²⁰⁸.



Figure 19: Diverse ranges of stiffness depending on tissues

All cells are subjected to mechanical forces. However, and depending on the tissue, these forces differs greatly. As a consequence, differentiated cells are tuned based on these differences to optimized their growth, survival and differentiation. Nonetheless, tumor development lead to stiffer matrix, as for breast tumor, responsible for malignant cues. Notably, other cell types, heatlhy ones, can be subjected to much higher stiffness, indicating the critical association between environment and tissue phenotype.

Source : Butcher, Alliston, and Weaver, 2009²⁰³

Regarding cancer, and more specifically breast cancer, the estimated value of normal stiffness of fat and mammary gland are respectively 17 and 160 Pa while it increases up to 4 kPa in breast tumor²⁰⁹. The stiffening of the extracellular matrix is mostly generated by collagen (major component of the ECM) cross-linking via the secreted protein lysyl oxidase (LOX)²¹⁰. This protein is frequently overexpressed in hypoxic tumors (i.e. deprived of oxygen) and initiates covalent collagen cross-linking, thereby increasing tension²¹¹. The major role of LOX in tumor

progression has been highlighted by Weaver and her colleagues in a report where, by artificially increasing collagen cross-linking in a mouse model, they observed not only stiffened tissues, but also an enhanced growth and invasion of non-cancerous cells. Reversely, inhibition of LOX impairs tumor progression²¹⁰.

Nevertheless, the connection between matrix stiffness and tumor progression is broadly accepted and supported by lots of studies^{207,212,213}. On top of that, mechanical changes of the ECM have been established as the cause of tumor progression through the mechanical signaling they generate²⁰⁶. Mechano-transduction will be address in part III.A.3. Several techniques are used to study the effect of stiffness on cells (Figure 20).



Figure 20: Experimental setups to analyze stiffness in vitro

Stiffness can be studied on cells seeded on coated substrates such as collagen or fibronectin (i), but also by plating them on gels of various stiffness (not shown). In three-dimensional models, cells can be grown as spheroids (ii) or as single cells (iii) embedded in a matrix. In all these models, rigidity can be tuned by changing the concentration of matrix proteins.

Source: Kalli and Stylianopoulos, 2018²⁰⁷

In two-dimensional (2D) experiments, cells can be plated on substrates coated with collagen or fibronectin, while three-dimensional (3D) models include single cells or tumor spheroids embedded in collagen or Matrigel. In both models, the matrix rigidity can be regulated by the use of an ECM-coated hydrogel (often polyacrylamide) of controlled stiffness (2D), by adjusting the protein density or the degree of cross-linking of the matrix (3D). 2D experiments based on the use of polyacrylamide gels of different stiffness^{214,215} have allowed researchers to initially

identify the role of matrix stiffness in mechano-transduction and other characteristics of tumors such as migration, proliferation or variance in gene expression.

2. Confinement

The other physical parameter to take into account during tumor development is confinement, or solid stress. This mechanical compression is the result of two forces: the first one is the external solid stress coming from the opposing force of the host tissue that counteracts tumor enlargement and the second one corresponds to the growth-induced solid stress accumulated within the tumor while growing²⁰³. In order to become larger, the tumor stiffness must be greater than the surrounding host tissue, at least 1.5 time bigger according to the model of Voutouri et al.²¹⁶. This allows the tumor to push against the normal tissue, hence making its own space to grow in size. Concerning the growth-induced stress, it keeps increasing during tumor growth while stiffening ceases²⁰⁶. At this point, the interconnection between solid stress and stiffness diverges. This distinction is demonstrate by Nia et al. in a study in which they compared stress amounts in primary tumors versus metastatic tumors in pancreas and colon cancers. Interestingly, both cancers exhibit opposite patterns whereas the stiffness remain the same. These findings revealed the uncoupling between solid stress and stiffness and provide a new insight of tumorigenesis and metastasis, thereby emphasizing the importance of studying separately these two parameters during further investigations^{206,217}.

A vast majority of studies have indeed focused on the ECM stiffness when considering the relationship between cancer progression and physical changes, while the role of solid stress remained underestimated. However, an increased number of studies have developed experimental systems mimicking the confinement observed in the tumor microenvironment (Figure 21)²¹⁸. In vivo measurements of brain, pancreatic, colon and breast tumors evaluate the growth-induced stress in the range of 0,21-20 kPa²⁰⁷.



Figure 21: Experimental methods to apply confinement on cells

In order to mimic solid stress in vitro, cells can be grown as spheroids, either in a matrix (i), but also by the use of capsules (ii). Spheroids can be confined by a polymer device as well (iii). Regarding two-dimensional experiments, a cell monolayer can be subjected to solid stress by the appliance of a weight directly on cells (iv), or in cells embedded in a matrix network (v). Another tool to study confinement, not mentioned here, is microchannel in which cell are forced to migrate through narrow tubes, and enables live-cell imaging.

Source: Kalli and Stylianopoulos, 2018²⁰⁷

In these setups, cells can be grown as spheroids either in agarose gel, imitating the external solid stress and the estimated solid stress is in the range of 6-16 kPa²¹⁹, or spheroids can be encapsulated, thus allowing a finer control of the spheroids' size²²⁰. Another mean by which tumor spheroids can be submitted to mechanical pressure is via the use of polydimethylsiloxane (PDMS) microdevices²²¹. These studies all show a mitotic arrest of the mechanically confined spheroids, thus indicating the activation of specific signaling pathways driven by solid stress. Tumor cells can also be compressed in 2D or in a matrix by using a piston of variable weight^{222,223}. In addition to the setups previously described, microchannel devices also mimic solid stress and allow real-time imaging of migrating cells under confinement²²⁴. These studies have revealed an increased apoptosis under high compression, while low compression (in the range of 0.29 to 0.77 kPa) enhances invasion, the expression of metalloproteinases and other metastatic associated genes²²⁵.

All together, these data imply a mechanical regulation of tumor growth and metastatic potential and this, by only considering solid stress, separately from the stiffness parameter.

B. Mechanotransduction

A broad range of cellular functions appears to be mediated by mechanical cues and, among them, several take part in cancer(Figure 22)²²⁶.



Figure 22: The influence of the extracellular matrix on the hallmarks of cancer

During tumor development, the extracellular matrix (ECM) influences each hallmark of cancer. The ECM is recognized by receptors at the cell surface and initiate pro-oncogenic cascades. Proliferation is induces via the Erk and PI3K pathways and the response to growth suppressors p15 and p21 is impaired as for the induction of apoptosis mediated by p53. Pro-migratory pathways are enhanced through Rho and Rac signaling while stiffness fosters angiogenesis. At all stages of tumorigenesis, the ECM promotes tumor progression by sending pro-cancerous cues.

Source: Pickup, Mouw and Weaver, 2014²²⁶

Cells are able to sense their surrounding environment and transmit these informations, which, in turn, regulate the cells' responses in order to adapt to different physical conditions. Both stiffness and solid stress have been demonstrated to promote tumor progression²⁰⁷. The principal changes observed involve tumor proliferation and migration/invasion. Mechanotransduction defines the ability of cells to translate mechanical stimuli into biochemical signals. This process is physically based on the force transmission between the extracellular matrix, the cytoskeleton and the nucleus. However, mechanotransduction can occur directly at the plasma membrane and be transmitted to the nucleus by several pathways²²⁷. Because integrins are able to transmit forces coming from the ECM to the cytoskeleton and also to regulate signaling pathways, they appear to be the most competent actors in mechanotransduction²²⁸. Integrins are able to cluster into two distinct structures involved in mechanotransduction: focal adhesions (FAs) and clathrin-coated structures (CCSs). However, these receptors are not the only mechanosensors at the cell surface, several studies have focused on the role of specific proteins (such as the Yes-associated protein YAP) but also on the ability of the cells to sense internal mechanical forces leading to rapidly adapted responses of the cell to these changes²²⁹.

1. Focal adhesions

When subjected to physical forces resulting in an increased adhesion, integrins aggregate and, in cooperation with numerous proteins, form focal adhesions (FAs). Within nascent FAs, integrins get activated and lead in turn to the stabilization of FAs via phosphorylation of the focal adhesion kinase (FAK) and cytoskeletal reinforcement. Mature FAs then perpetuate downstream signaling and cytoskeletal remodeling by the activation of signaling pathways such as Ras, Rac and Rho^{230,231}. It is noteworthy that integrins can interact with other receptors at the plasma membrane and these crosstalks, for instance with EGFR, have been observed in cancerous processes such as proliferation or migration²⁰⁹. Moreover, both signaling of FAK and growth factor receptors are regularly upregulated in cancer, hence highlighting the particular relevance of these proteins in a pathophysiological context²³². Moreover, the role of mechanical stress as a driver of focal adhesions growth and maturation is well established^{233,234}.

In order to keep growing, tumor cells have developed three survival mechanisms: sustained proliferation, evasion to growth suppressors and apoptosis escape. An increased collagen matrix density is responsible of higher cell proliferation (Figure 23)²³⁵.



Figure 23: High collagen density induces more proliferation

(a) MCF10A human mammary epithelial cells seeded for 21 days in low (1.3 mg/ml) or high density (3.0 mg/ml) stained for actin (green) and nuclei (blue). Well-organized acini structures are form in low density matrices (left panel) while disorganized cell colony is generated in high density matrix (right panel). (b) Amount of proliferating cells are measured by Ki67 antigen detection and reveal higher level of proliferation of cells within high density matrices.

Source: Provenzano et al., 2008²³⁵

Adhesion to a substrate is necessary for cell cycle progression²³⁶. Focal adhesions and FAK phosphorylation activate Ras and phosphatidyl-inositol-3-kinase (PI3K) signaling which in turn activate the extracellular regulated mitogen activated protein kinase (MAPK) Erk²³⁷. Erk then translocate into the nucleus and promotes cell proliferation through the induction of cyclin D1 and the downregulation of cell cycle inhibitors. Mechanical stress caused by tumor growth and matrix stiffening increases cell adhesion, thus providing tumor cells the mean to keep proliferating and evade apoptosis^{210,238}. However, cells submitted to high levels of confinement often stop proliferating and undergo apoptosis, while low levels of confinement induce migration and invasion^{219,221,225}.

2. Clathrin-coated structures

In addition to FAs, clathrin-coated structures, due to their ability to cluster integrins and also to form signaling platforms, also behave as mechanosensing structures¹⁷⁶.

Similarly to FAs pathways, proliferation can also be triggered by clathrin-coated structures which, by strongly binding the ECM, especially in the case of high substrate stiffness, lose their ability to perform endocytosis. As a consequence, many signaling receptors accumulate in these frustrated clathrin structures and, instead of being internalized, keep signaling at the plasma membrane, leading to strong downstream Erk signaling and an enhanced proliferation¹⁷⁶.

Regarding confinement, CCSs also appear to be mechanosensors as previously shown in the paper from Ferguson et al.¹⁹⁵. By applying different mechanical stresses such as microaspiration, confinement and hypo-osmotic chocs, Ferguson and colleagues showed that clathrin-coated structures become less dynamic. However, they did not dig further to analyze and understand the underlying mechanism of this phenotype.

3. Mechanosensitive signaling pathway (YAP)

Signals associated with mechanically-induced modifications of the cytoskeleton through integrins also converge to the Yes-associated protein (YAP). This protein and the related protein TAZ, are transcription factors remaining in the cytoplasm under normal conditions, but when activated, translocate to the nucleus and activate gene implicated in tumor growth²³⁹. YAP/TAZ nuclear activity correlates with the stability of the cytoskeleton and cell tension, mediated upstream by FAs signaling via the Rho pathway.²⁴⁰ YAP is also important in the activation of cancer-associated fibroblasts (CAFs)²⁴¹. These CAFs promote tumor progression by producing soluble factors and remodeling the ECM²⁴². In other words, matrix stiffening enhances YAP activation which in turn creates a mechanosensitive positive feedback loop by maintaining the CAF phenotype and hence, amplifying ECM rigidity.

Importantly, it has also been proven that under confinement, nuclear pores open and enable YAP to translocate from the cytosol to the nucleus without any requirement for other upstream

signals²⁴³. These evidences demonstrate the role of YAP/TAZ proteins which behave as mechanosensors, downstream of FAs signaling or via force-induced nuclear entry directly.

4. Nucleus

Emerging evidences revealed the nucleus as a mechanosensory organelle itself. Forces transmitted across the cytoskeleton lead to nuclear deformation and modifications of gene expression as well as nuclear envelop structure and composition modulations²⁴⁴. This suggests the active participation of the nucleus in sensing changes in mechanical load. The main component of the nuclear envelope is the lamin A. Alterations of this protein have been linked to diseases called laminopathies, thus demonstrating the crucial role of lamin in preserving the structural integrity of the nucleus. Moreover, lamin A levels have been proven to scale with tissue elasticity²⁴⁵. Indeed, tissue stiffness increases expression of lamin A, suggesting an active feedback between mechanical changes in the microenvironment and nuclear properties, probably to preserve DNA integrity.

5. Migration/invasion

The other important consequence of mechanical changes is their effect on cell migration and invasion. Some cell types are able to sense and follow stiffness gradients of the extracellular matrix through focal adhesions, and this process is called durotaxis^{246,247}. Durotactic behaviors have been observed in development, fibrosis and cancer^{248,249}. Fibroblasts migrate to stiffer environments and this includes tumor microenvironment where they get activated and promote migration and invasion of cancer cells.



Figure 24: Single cell motility modes: mesenchymal migration versus amoeboid movement

Cells are able to migrate in two main distinct fashions. The most common is the mesenchymal mode of migration (left panel). This requires adhesion to the substrate through the formation of focal adhesions (Fas) at the leading edge of the cell which promote cell contractility and propel the cell in a determined direction. The movement is coupled with matrix degradation at the front of the cell in order to create enough space for the cell to migrate. The second motility mode is the amoeboid-like way. In this case, instead of forming protrusions coupled with matrix degradation, the cell adopts a round shape with dynamic blebs. This mode of migration is allow the cell to squeeze through narrow spaces formed by the surrounding matrix and thus do not require adhesion nor matrix degradation.

Source : adapted from Alexandrova, 2014²⁵⁰

Tumor cells exhibit two distinct invasive behaviors: a mesenchymal-like type of migration or an amoeboid-like type of migration (Figure 24)^{251,252}. Depending on the cell type, cells display one or the other mode of migration and this modes are mutually interchangeable and both phenotypes are implicated in mechanical response²⁵³. To better understand these ways of moving, it is important to place the matter in the proper context. The mesenchymal type of motility is based on the formation of long membrane actin-rich protrusions at the leading edge of polarized cells, termed lamellipodia, which require the formation of FAs linked to the matrix. This process is driven by Rac signaling. At the same time, the integrin clustering recruits ECM proteases to degrade the ECM and create paths through this dense environment. Then the cell body translocates towards the leading edge of the cell strongly bound to the ECM. The resulting movement is slow, directional, and based on alternating pushing and pulling cycles. This mode of migration is encounter in fibroblasts, keratinocytes, endothelial cells and some tumor cells. During cancer development, matrix stiffness generates increased adhesion forces via focal adhesions and can thus promotes this mode of migration^{254,255}.

The other mode of migration is the amoeboid-like phenotype. Cells displaying this type of motility have the particularity to, instead of degrading the ECM, squeeze their bodies through the gaps in the ECM network. This invasive strategy relies on cycles of expansion and contraction which propels cells throughout pores of the ECM network. In order to do so, cells adopt a more round shape with a cortical distribution of the actin cytoskeleton and do not strongly adhere to the substrate. Indeed, both modes of migration are easily discernable by eye (Figure 24). Amoeboid mode of migration is dependent of Rho/ROCK signaling pathway and exhibits a high velocity compared to mesenchymal migration. Cell migrating in amoeboid fashion are typically leukocytes and, sometimes, some cancer cells^{251,256}. However, these two migration mechanisms are interchangeable and the switch to the other mode of invasiveness is either called mesenchymal/amoeboid transition (MAT) or amoeboid/mesenchymal transition (AMT)²⁵⁷. MAT can, for instance, be induced by inhibiting matrix degradation^{258,259}. The other parameters taken into account in transition of migration are, as discussed before, adhesiveness, cell contractility and matrix stiffness²⁶⁰.

Some studies have focused on migration in restricted spaces²⁶⁰. They demonstrated that, when subjected to solid stress, cells undergo the mesenchymal/amoeboid transition^{261,262}. However, numerous cues can influence the mode of migration used by confined cells. These determinants firstly comprise the pore size of the matrix. Indeed, a decisive aspect of migration under confinement is the size and stiffness of the nucleus, which, because of its rigidity, is the biggest limiting factor potentially impeding cell migration through ECM pores^{263,264}. As solid stress

strengthens, it becomes more laborious for tumor cells to squeeze enough their nucleus in order to migrate through narrower spaces. If gaps are too tighten, nuclear translocation can't take place and migration stops unless the matrix get remodeled or degraded by the cell itself.

Tumor formation and progression are closely related to the tumor microenvironment. The role of the surrounding environment on cancer cells have been highlighted in this section. Nevertheless, signals coming from the microenvironment can also cause genetic instability, which makes it a potent carcinogen²⁶⁵. The accumulation of genomic alterations, due either to mutations, chromosomal rearrangement or impairment of DNA repair mechanisms and tumor suppressors promotes tumor development and malignant transformation. Among these alterations and because of its tight connection to integrins and endocytosis, a peculiar focus on the epidermal growth factor receptor will be treated in the section below.

IV. The epidermal growth factor receptor

Beginnings of the current knowledge about the epidermal growth factor receptor (EGFR) started with the isolation of a protein responsible of epidermal growth and keratinization, the so-called epidermal growth factor (EGF)²⁶⁶. The identification of its receptor, the EGFR, ensued a decade later²⁶⁷. Since these pioneering discoveries, lots of studies have been conducted to decipher the mechanisms underlying the activation and the pathways responsible for the observed variations of cellular behaviors mediated by EGFR activation²⁶⁸. These modifications include cell proliferation, survival and differentiation.





Schematic representation of the EGF receptor (EGFR). The EGFR is a transmembrane receptor with an extracellular part dedicated to ligand recognition and an intracellular element comprising the tyrosine kinase domain and the C-terminal tail with tyrosines that can be phosphorylated. At steady state, the EGFR adopts a closed conformation and convert to an activated form and dimerized upon the addition of its ligand (EGF or EGF-like ligands). Simultaneously at the cytoplasmic part, kinases are activated and lead to the transphosphorylation of the tyrosine residues at the tail, hence initiating signaling cascades.

Source : Huang et al., 2016²⁶⁹

The epidermal growth factor receptor (EGFR), also called ERBB1, is one of the four members of the ErbB family of receptor tyrosine kinases (RTKs) along with ERBB2 or HER2, ERBB3 (HER3) and ERBB4 (HER4). All members present an extracellular ligand-binding region, a single transmembrane region and a cytoplasmic tyrosine-kinase (TK) containing domain (see Figure 25 for the EGFR specific architecture).

Regarding EGFR, the extracellular region includes four domains (I, II, III and IV): domains I and III constitute the ligand-binding domain, connected by the domain II which presents the dimerization arm. These three domains are referred as the "head" of the receptor, while the domain IV composes the "leg".

Under normal physiological conditions, ERBB receptors get activated by their ligands, which belong to the EGF family of growth factors²⁷⁰. In the absence of ligand, the extracellular region adopts an autoinhibited conformation²⁷¹. Ligand binding remodels the shape of the receptor into an active straightened conformation which makes the dimerization arm accessible and hence induces the formation of back-to-back homo- or heterodimers. This dimerization, together with a series of conformational changes, leads to the asymmetric dimerization of the cytoplasmic domain, stabilized by the juxtamembrane segment, and thus activation of the intracellular tyrosine kinase domains. This enzyme activation enables the auto- and transphosphorylation of tyrosine residues, specifically Y845, Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, Y1173, at the C-terminal cytoplasmic tail and initiates the downstream signaling²⁷². This ligand-dependent signalization is the best described and referred as the canonical EGFR pathway.

A. Ligands

The downstream signaling of the EGFR is highly dependent of its ligand. Indeed, seven ligands with the ability to bind the EGFR have been identified. Alongside epidermal growth factor (EGF), the best characterized EGFR ligand, transforming growth factor a (TGF-a), amphiregulin (AREG), epiregulin (EREG), epigen (EPI), betacellulin (BTC) and heparinbinding EGF-like growth factor (HB-EGF) all have the potential to bind the EGFR. Among them, only EGF, TGF-a and AREG are specific to EGFR. However, all of them are first synthetized as transmembrane precursors that can be proteolytically cleaved to generate soluble ligands. The "a disintegrin and metalloproteinase" (ADAM) family of metalloproteases and more specifically ADAM10 regarding EGF and BTC, and ADAM17 for EREG, TGF-a and HB-EGF appear to be the major proteins responsible for the shedding of these ligands²⁷³. In some circumstances, membrane-anchored ligands, in addition to soluble ones, may behave as biologically active ligands. Therefore, activation of the EGFR upon binding of its ligands has been observed in juxtacrine, autocrine, paracrine and/or endocrine manner²⁷⁴. In juxtacrine signaling, non-cleaved ligands on the inducing cell interact with receptors of adjacent cells as for TGF-a, HB-EGF and AREG whereas autocrine, paracrine and endocrine signaling require the cleavage of transmembrane ligands.

B. Trafficking

Endocytosis is the major regulator of RTK signaling. Many studies have observed distinct biological responses depending on the ligand^{275,276}. Yet, all ligands generate the same sequence of events on EGFR, comprising ligand binding, EGFR activation, dimerization and intracellular phosphorylation (this last step also contributes to specific responses, see section below). Following these events, active EGFRs are removed from the plasma membrane through endocytosis. EGFR internalization is mediated by Cbl, an E3 ubiquitin ligase permanently bound to activated EGFR and several other receptors to promote continuous multi-ubiquitination of these receptors²⁷⁷. Attachment of ubiquitin molecules fates the receptor for

degradation into lysosomes. Nonetheless, EGFR itself is an active actor of its own endocytosis. Indeed, EGFR itself can indirectly controls clathrin-assembly through downstream activation of the Src kinase which phosphorylates the clathrin heavy chain (CHC)²⁷⁸. Moreover, EGFR is also able to mediate Eps15 phosphorylation, essential for endocytosis, but also monoubiquitination of Eps15 and epsin to degrade them^{279,280}. In addition, mono-ubiquitinated epsin is unable to bind to PIP₂ neither to interact with AP2 and clathrin which, as a consequence, reduces EGFR endocytosis. EGFR is therefore a receptor with the ability to control its own destiny.

The differences observed in biological responses would rely on ligand-receptor affinity instead of differential EGFR activation. Ligand affinity already differs among EGFR ligands and can also be affected by the association of EGFR as homo- or heterodimers and this have a clear impact on the receptor trafficking and signaling. Indeed, strength of the ligand-receptor interaction guides the endosome sorting of the internalized-EGFR, which relies on the dissociation or not of the ligand from the receptor in the endosomal compartment, hence favoring recycling or degradation respectively²⁸¹. These different routings mediate either further signaling at the plasma membrane after recycling or signal termination in the case of degradation. Nonetheless, although CME was originally thought to terminate the EGFR signaling process, increasing evidences pointed out to a sustained signaling in endosomes¹⁶⁰.

C. Signaling pathways

The first reaction to the EGFR intracytosolic phosphorylation is the recruitment and activation of downstream signaling molecules that are able to bind phosphotyrosine (pYs) residues. The first recruited proteins get phosphorylated by the EGFR kinase domain and can act as docking proteins to recruit further signaling proteins. These proteins contain a phosphotyrosine binding (PTB) domain or Src homology domain (SH2)²⁸² (Figure 26).



Figure 26 : Epidermal growth factor receptor interactome via phosphorylated residues

The left panel represents the known proteins interacting with the activated EGF receptor. All these proteins display either Sarc homology 2 (SH2) domain or phosphotyrosine binding (PTB) domain. They can be separated in three groups: enzymes, regulators and docking proteins. Unlike enzymes, regulators and docking proteins lack intrinsic catalytic domains but provide a physical bound between EGFR and other effector proteins. The right panel illustrates the intracytosolic part of the EGFR and especially the phosphorylation sites at the C-terminal tail. This includes Y845, Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148 and Y1173. A few examples of interacting proteins are shown here such as Src homologous and collagen (Shc) protein with pY992, pY1148 and pY1173, but also growth factor receptor-bound protein-2 (GRB2) with pY1068 and pY1086 and finally the phospholipase C γ (PLC- γ) protein bind pY1173.

Source : adapted from Foreman, Johansen, and Gibb, 2011²⁸³; Conte and Sigismund, 2016²⁸⁴

Nine tyrosine residues at the cytoplasmic tail of the EGFR are potent for phosphorylation, allowing multiple interactions with docking proteins which can influence a large number of signaling effectors²⁸⁵. From there, different signaling cascades can be initiated (Figure 27). The specificity of the cascade activated is determined by the ligand, the positive or negative effectors of the receptor, but mostly by which tyrosine residues are phosphorylated (ligand-dependent and dimer-dependent).



Figure 27: Erbb signaling from the cell surface and consequences on the cell physiology

(a) All the known ligands for different ErbB combinations. Numbers indicated in ligands blocks represent a high affinity of the ligand for the designated receptor. Ligands specificity is only point out for the epidermal growth factor (EGF) and neuregulin 4 (NRG4). No ligand displays a high affinity for ErbB2 and ErbB3 is catalytically inactive. ErbB regulation can also happen under G-protein-coupled receptors (GPCRs) and cytokine receptors. (b) Signaling pathways are triggered by ErbB activation. The EGFR homodimerization stimulates a vast majority of signaling cascades. (c) These activated pathways lead to various responses from the cell, including pro-oncogenic ones such as migration, growth or adhesion.

Abbreviations: Abl, a proto-oncogenic tyrosine kinase; Akt, a serine/threonine kinase; GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, Janus kinase; PI(3)K, phosphatidylinositol-3-OH kinase; PKC, protein kinase C; PLC γ , Phospholipase C γ ; Shp2, Src homology domain-2-containing protein tyrosine phosphatase 2; Stat, Signal transducer and activator of transcription; RAF–MEK–MAPK and PAK–JNKK–JNK, two cascades of serine/threonine kinases that regulate the activity of a number of transcription factors; SOS, Son of sevenless

Source : Yarden and Sliwkowski, 2001 286

Among all the cascades potentially stimulated, the Ras- and Shc-activated mitogen-activated protein kinase (MAPK) pathway is constantly triggered regardless of the ligand. The

phosphoinositide-3-kinase (PI3K)/Akt pathway is activated downstream of most dimerization events, and its kinetics depends on which ErbB receptor is involved since PI3K binds directly to ErbB3 and 4, but indirectly to EGFR and ErbB2²⁸⁵. Besides these two major pathways, the phospholipase C (PLC), the Janus kinase/signal transducers and activators of transcription (JAK/STAT), and the c-Jun N-terminal kinase (JNK) cascades can be initiated upon ErbB receptors activation as well²⁸⁶. Each receptor is associated to a specific array of signaling proteins, but some of these cascades are shared by different ErbB members as shown in the figure above (Figure 25). All together, these cascades govern critical cellular processes such as cell survival, proliferation, migration, adhesion, apoptosis, and/or differentiation. Given the role of ErbB signaling network in mediating the previously mentioned cellular mechanisms, dysregulations of ErbB pathways are actually observed in a wide range of cancers, which seems quite expected.

D. EGFR and cancer

The EGF receptor was the first ErbB member to be associated with human tumors^{287,288}. The ErbB family of receptors is subjected to various alterations in cancer. In many types of cancer, the ErbB signaling network is found hyperactivated due to overexpression or constitutive activation of receptors, overproduction of their ligands, but also upregulation of their downstream effectors²⁸⁹. Moreover, mutations leading to the silencing of the most important negative regulator of the cell-survival pathway downstream of PI3K, namely the phosphatase and tensin homolog (PTEN) can also contribute to tumorigenesis²⁹⁰.

Overexpression of EGFR is a way to promote sustained signaling. Indeed, internalization through clathrin-mediated endocytosis is limited due to the availability of adaptor proteins. Consequently, EGFR endocytosis is slowed and preferentially followed by recycling which significates more signaling at the plasma membrane²⁹¹. In addition to EGFR, ErbB2 is also frequently upregulated in cancers. This overexpression leads to heterodimerization with EGFR

and induces the recycling of both receptors to the cell surface, in part by escaping ubiquitininduced degradation²⁹².

High levels of a mutant form of EGFR is found in different cancer types such as glioblastoma and head and neck squamous cell carcinoma (HNSCC). This mutant, called EGFRvIII, is a deletion mutant lacking exon 2 to 7 which includes a part of the ligand-binding domain. However, this mutant form of EGFR is able to spontaneously dimerized and get activated in the absence of ligand. Moreover, and unlike EGFR, EGFRvIII is improperly degraded in lysosomes, instead leading to sorting for recycling^{293,294}. The expression of this deletion mutant hence confers a high tumorigenic potential to the cells expressing it.

Moreover, somatic mutations in the tyrosine-kinase domain of EGFR have been reported in non-small-cell lung cancers (NSCLCs) patients under treatment with tyrosine kinase inhibitors (TKIs) and more precisely Gefitinib²⁹⁵. One mutation (replacement of leucine by arginine at codon 858 [L858R]) of EGFR was initially described as linked to a high responsiveness to this treatment. However, nearly all patients presenting this high sensitivity to gefitinib eventually relapse after a certain time under treatment. When researchers sequenced de novo their EGFR gene, they revealed the presence of a second mutation (replacement of threonine by methionine at codon 790 [T790M]). Construction of this mutant EGFR in cells in vitro revealed the causal link of this specific mutation in drug resistance²⁹⁶.

Regarding ligands alterations, the TGF- α is the best characterized in human cancers. TGF- α is co-expressed with EGFR is several carcinomas, such as lung, ovary or colon cancers and is associated with a poor prognosis²⁹⁷. In the case of prostate cancer, TGF- α expression was first observed in the tumor stroma while it was synthetized directly by tumor cells at later stages of tumor progression²⁹⁸. Expression of TGF- α , a ligand with low affinity for the EGFR also participates to cancer evolution. Indeed, and as said before, the low ligand affinity renders it more sensitive to pH and favors dissociation in the endosomal compartment. Ligand disengagement fosters routing for recycling of the EGFR back to the plasma membrane. Overproduction of TGF- α hence promotes constitutive proliferation by supplying a continuous source of ligands which prevents receptor down-regulation²⁹⁹.
On top of that, emerging roles of the EGF receptor as a mechanosensitive protein have been highlighted in previous studies. As a matter of fact, EGFR is able to interact with integrins in certain conditions and promote cell spreading and migration (Figure 28)^{300,301}.



Figure 28: Crosstalk between epidermal growth factor receptor and integrins

Integrins are able to cooperate with other receptors at the cell surface, such as EGFR or G protein-coupled receptors (GPCRs). First, integrins can promote Src-mediated phosphorylation of EGFR in the absence of its ligand (not shown) and this occurs via the signaling complex Src, p130Cas. Ligand-induced activation of EGFR activates Src directly and enhances not only integrin signaling involving the FAK-Src complex, but also the common EGFR signaling cascade which comprises the Ras/Erk pathway. A third actor can participate to this reaction, GPCRs. Indeed, chemokines in the surrounding medium can activate these receptors and thus promote integrins expression at the cell surface. All together, these crosstalks enhance the malignant transformation of cells.

Source : Desgrosellier and Cheresh, 2010³⁰⁰

Integrin-mediated adhesion leads to Src activation which in turn phosphorylates EGFR on specific residues in the absence of ligand. However, the pattern of EGFR phosphorylation appears to be different upon integrins activation from the one induced by its ligand, the EGF³⁰².

Such crosstalk between integrins and EGFR initiates PI3K cascade and reach the guanine nucleotide exchange factor for Rho family, namely Vav2 and stimulates lamellipodia formation³⁰³. Cell spreading and migration are already known to be consequences of rigidity sensing which affects integrins activity. Based on these observations, the group of M. Sheetz focused on the role of EGFR as a mechanosensor protein³⁰⁴. They uncovered the involvement of EGFR for rigidity sensing on stiff surfaces only, when they associate with nascent focal adhesions forming in response to stiffness.

Moreover, EGFR can also behave as an indirect mechanosensory protein. Previous studies underlined a shedding of cleaved-EGFR ligand under mechanical strain. Tschumperlin and colleagues results demonstrate an autocrine release of EGFR ligands and hence, EGFR activation by compressive stress applied on bronchial epithelial cells³⁰⁵. Since then, these results have been supported by a further study during lung development³⁰⁶. In this model, some metalloproteases are activated by the compressive stress and thus cleave HB-EGF, resulting in the autocrine EGFR activation. Although it is not clear which metalloprotease exactly is involved, neither how compression activates this protease, the process can be inhibited by Batimastat, a large spectrum metalloproteases inhibitor^{307,308}.

PHD OBJECTIVES

Our growing understanding of non-canonical roles of clathrin-coated structures at the plasma membrane highlights their importance in the cell response to physio-pathological conditions. Indeed, recent advance in our team focused on the presence of long-lived clathrin structures which act as signaling platforms and initiate MAPK/Erk mediated proliferation. The appearance of these long-lived CCSs, also termed clathrin-coated plaques, increases when the cells are grown on a stiff substrate¹⁷⁶. The formation of these structures results from a frustrated endocytosis process whereby the budding force exerted by the clathrin coat is counteracted by an adhesion force mediated by an integrin linking the clathrin coat to the substrate. As discussed in the introduction, other mechanical parameters can also potentially frustrate CCSs, like an increased membrane tension.

Many studies have already examined cells under confinement and its effect on cell behaviors, such as migration and invasion²⁴³. Although they revealed a strong correlation between solid stress and enhanced cell movement associated with a switch from a mesenchymal to amoeboid moving fashion, most of these studies concentrate at one cell at a time²⁶². As a matter of fact, the methods employed, including microchannels or Atomic force microscopy (AFM), are designed for live-cell imaging and don't permit the study of a big amount of cell at once. As a result, a research conducted on clathrin-coated structures as in cells under confinement have revealed an alteration of their dynamics but researchers weren't able to dig further because of the method employed¹⁹⁵.

In order to solve this problem, I used and adapted another method consisting of a cell culture squeezed under an agarose gel³⁰⁹. After setting up this technique, we were able to perform both imaging and molecular experiments on confined cells. In this manner, measurement of CCSs dynamics and protein recruitment to them were achievable by microscopy while activation of downstream signaling by quantifying proteins levels was made also possible.

The aim of my PhD project was to investigate the participation of clathrin-coated structures in cell response to confinement. In particular, we wanted to analyze the dynamics of CCSs under confinement and determine whether they become frustrated, as observed with other types of mechanical perturbations. Also, because frustrated CCSs serve as signaling platform for different receptors, we wanted to determine if this was also the case under confinement and what receptor(s) was involved in the cell response to confinement.

Title:

Endocytosis frustration potentiates compression-induced receptor signaling

Authors:

Dahiana Le Devedec^{1*}, Francesco Baschieri^{1*}, Nadia Elkhatib¹, and Guillaume Montagnac¹

Affiliations:

¹ Inserm U1170, Gustave Roussy Institute, Université Paris-Saclay, Villejuif, France

* These authors contributed equally

Corresponding author: guillaume.montagnac@gustaveroussy.fr

Abstract:

Cells respond to environment-induced mechanical perturbations by many different means. Clathrin-coated structures (CCSs) are sensitive to such perturbations in a way that often results in a mechanical impairment of their capacity to bud, thus preventing endocytosis. Compressive stresses can be exerted in different physiological and physio-pathological contexts and elicit specific responses that help the cell to cope with the stress. Here, we show that compression leads to CCSs frustration that is required for pressure induced-epidermal growth factor receptor (EGFR) signaling. We confirmed that pressure stalls CCSs dynamics and showed that it also slows down the dynamic exchange of CCSs building blocks. EGFR specifically accumulated at frustrated CCSs under pressure, while other receptors did not, in the absence of any added specific ligands. Surprisingly, compression-induced EGFR recruitment at CCSs was independent of EGFR kinase activity, but CCSs were required for full EGFR activation and signaling. Finally, we observed that compression-induced CCSs frustration can also potentiate signaling through other receptors, provided their ligands are present in the environment. We propose that pressure modulates intracellular signaling events partly through generating frustrated CCSs.

Main Text:

INTRODUCTION

Clathrin-mediated endocytosis (CME) relies on the assembly of clathrin-coated structures (CCSs) at the internal leaflet of the plasma membrane. CCSs are endowed with the capacity to recruit specific receptors and to bend the membrane in order to generate receptor-containing endocytic vesicles¹. Membrane bending is however sensitive to mechanical perturbations that oppose the invagination force generated by CCSs. For instance, high membrane tension was reported to stall CCSs invagination and thus, to prevent CME². Other types of mechanical perturbations can also prevent normal CCSs budding. For example, a subset of CCSs termed tubular clathrin/AP-2 lattices (TCALs) that specifically nucleate at cell/collagen fibers contact sites show a reduced dynamics because they try and fail to internalize fibers that are longer than the cell itself³. Substrate rigidity can also impair CCSs budding through favoring the $\alpha\nu\beta$ 5 integrin-dependent formation of flat and long-lived clathrin-coated plaques⁴. Thus, CCSs frustration is a common response to a wide array of mechanical perturbations. CCSs frustration may not simply be a passive consequence of environmental perturbations but may actually participate in building an adapted response to these modifications. Indeed, we showed that TCALs help the cell to migrate in 3D environments and that clathrin-coated plaques that assemble on stiff substrates serve as signaling platforms for different receptors, thus leading to sustained cell proliferation^{3,4}.

Cell compression was recently shown to induce CCSs frustration as well, most likely because of an increased membrane tension that is believed to result from the compressive stress⁵. Compressive forces are frequently encountered in the organism, whether in a physiological or pathological context^{6–8}. These forces deeply impact the cell physiology and modulate signaling pathways as well as gene expression profile⁹. Compression was shown to lead to the activation of the epidermal growth factor receptor (EGFR) through a force-induced shedding of HB-EGF precursor¹⁰. Because we previously observed that the EGFR uses frustrated clathrin-coated plaques as signaling platforms, we wondered whether pressure-induced CCSs frustration could participate in EGFR signaling in these conditions.

RESULTS

Compression reduces CCS and CCS component dynamics

To investigate the consequences of compressive forces on CCSs dynamics, we used HeLa cells that were genome-edited to express a GFP-tagged version of µ2-adaptin, a subunit of the clathrin adaptor AP-2. These cells were grown on glass coverslips and confined under an agarose plug. We noticed that confinement induced an enlargement of the cell area and blebs were often observed at the cell edges (Supplementary Fig. 1a), suggesting that membrane tension is most likely dramatically increased in these conditions¹¹. In addition, nuclei were enlarged as well under compression and nuclear blebs were also visible at their rim (Supplementary Fig. 1b). These observations indicate that cells are indeed experiencing compression in our assays. In classical culture conditions, HeLa cells display a mixture of canonical, dynamic CCSs and static clathrin-coated plaques. We observed that compression globally increased the lifetime of CCSs as well as the occurrence of static (lifetime >300s) CCSs (Fig. 1a and b), thus confirming previous report⁵. Because integrin $\alpha \nu \beta 5$, which is necessary for clathrin-coated plaque assembly, could possibly play a role in pressure-induced global loss of CCSs dynamics, we treated cells with Cilengitide, a potent $\alpha\nu\beta5$ inhibitor. While CCSs were mostly dynamic in Cilengitide-treated cells before pressure, confinement under the agarose gel dramatically increased the lifetime of CCSs as well as the occurrence of stalled CCSs (Fig. 1a and c). These results indicate that CCSs increased lifetime/stabilization under compression is independent of $\alpha\nu\beta5$ integrin and is most likely the consequence of increased membrane tension. Membrane tension was recently shown to regulate the dynamics of CCSs components in yeast^{2,12,13}. To investigate whether pressure also impacts the dynamics of major CCSs building blocks in our system, we performed fluorescence recovery after photobleaching (FRAP) experiments in cells expressing GFP-tagged µ2-adaptin. We chose to FRAP individual CCSs corresponding to clathrin-coated plaques because the long-lived nature of these structures allows to monitor fluorescence recovery over minutes. In control conditions, fluorescence recovery was fast (half-time recovery, $t1/2 \approx 8s$) with a plateau reaching approximately 80%, thus showing that only ~20% of AP-2 complexes were immobile at CCSs (Fig. 1d and e). However, the immobile fraction only reached approximately 60% and half-time recovery was delayed when pressure was applied on cells ($t1/2 \approx 15s$; Fig. 1d and e). These results show that cell compression slows down AP-2 turnover in a similar manner as increased membrane tension¹⁴.

Compression leads to CCSs-dependent EGFR signaling

Compressive forces have been reported to activate EGFR and downstream Erk signaling¹⁵. Indeed, we observed that compression triggered transient Erk activation (Fig. 2a and b). We also observed that GFP-tagged Erk transiently translocated from the cytoplasm to the nucleus when HeLa cells were confined under the agarose plug thus confirming the activation status of Erk in these conditions (Supplementary Fig. 2a and b). However, the mechanoresponsive transcription regulator Yes-associated protein (YAP) was excluded from the nucleus under pressure suggesting that this pathway is not activated in these conditions (Supplementary Fig. 2c). Pressure-induced Erk activation was dependent on EGFR expression (Fig. 2c and d) as well as on EGFR kinase domain activity as Gefetinib treatment inhibited Erk phosphorylation under compression (Fig. 2e and f). CCSs have been shown to act as platforms that potentiate receptor-mediated signaling, particularly in the case of the EGFR^{10,11}. CCSs lifetime is an important regulator of receptor signaling output and for instance, long-lived clathrin-coated plaques are more potent than dynamic clathrin-coated pits in supporting signaling pathway activation.

EGFR-dependent signaling in the Erk pathway. Indeed, we observed that AP-2 subunits or clathrin heavy chain (CHC) knockdown reduced Erk activation under compression (Fig. 2g and h). Thus, CCSs are required for full EGFR signaling in compressed cells.

Mechanisms of EGFR recruitment at frustrated CCSs under pressure

We next analyze if and how EGFR is recruited at CCSs. Genome edited HeLa cells expressing mCherry-tagged, endogenous µ2-adaptin and overexpressing GFP-tagged EGFR were compressed under an agarose plug and monitored using total internal reflection fluorescence (TIRF) microscopy. EGFR quickly accumulated at CCSs upon compression (Fig. 3a and b). EGFR activation and recruitment at CCSs are both believed to depend on ligand-induced dimerization of the receptor¹⁶. Yet, pressure led to EGFR activation while no specific ligand was added in the culture medium and we observed that compressing cells in the absence of serum did not prevent EGFR accumulation at CCSs nor Erk activation (Supplementary Fig. 3a and b). It has been reported that compression induces ectodomain shedding of the EGF-family ligand heparin-binding EGF (HB-EGF), thus leading to autocrine EGFR stimulation¹⁰. HB-EGF shedding is regulated by matrix metalloproteases whose inhibition was reported to prevent EGFR activation following compressive stresses¹⁰. Indeed, we observed that Batimastat, a potent and large spectrum inhibitor of matrix metalloproteases, strongly reduced EGFR recruitment at CCSs under pressure (Fig. 3b and c) as well as Erk activation (Fig. 3e and f). However, Gefitinib did not prevent the pressure-induced EGFR accumulation at CCSs (Fig. 3g and h). These results suggest that ligand binding, but not activation, is required for EGFR recruitment at CCSs. These observations are thus in favor of a model whereby ligand-induced EGFR dimerization is required to interact with the CCSs machinery, in a kinase domain activity-independent manner¹⁷.

Compression-induced CCSs frustration modulates receptor sorting and signaling

We next aimed at determining whether other receptors could also be recruited at CCSs upon cell compression. We first looked at different receptors whose endocytosis is normally triggered by their ligands. β1-adrenergic G-protein coupled receptor (GPCR), GPCR clathrin adaptor β-arrestin 2, as well as hepatocyte growth factor receptor (HGFR) which are all known to be recruited at CCSs upon stimulation^{18,19} did not accumulate at CCSs under pressure (Fig. 4a-c). These results indicate that compression does not result in the activation of these receptors in the absence of their specific ligand. We next analyzed the dynamics of the transferrin receptor (TfR) that is usually constitutively recruited at CCSs in order to be internalized. While GFP-tagged TfR strongly accumulated at CCSs in control cells, it was excluded from CCSs in compressed cells (Supplementary Fig. 4a and b). These surprising results suggest that compression modulates receptor sorting at CCSs. Along this line, we observed in FRAP experiments that fluorescence recovery of EGFR-GFP at CCSs was reduced in cells experiencing compression as compared to uncompressed cells stimulated with 10 ng/ml EGF (Supplementary Fig. 4c). Thus, compression impacts on both CCS component dynamics (Fig. 1d) and CCS cargo dynamics.

We next reasoned that compression-induced CCSs frustration could impact receptor signaling besides the specific case of EGFR. Indeed, CCSs lifetime has been reported to positively correlate with strong signaling output²⁰. Using HGF-supplemented medium, we observed that GFP-tagged HGFR did not obviously accumulate at CCSs in these non-acute stimulation conditions (Fig. 4d and e). However, GFP-HGFR was efficiently recruited at CCSs upon compression (Fig. 4d and e). This most likely results from low level HGFR activation in these non-acute stimulation conditions, leading to its progressive accumulation in frustrated CCSs

that cannot support anymore its endocytosis. We noticed that Erk was activated in these conditions, even if the classical compression-induced EGFR activation was prevented by Gefetinib treatment (Fig. 4f and g). This demonstrates that, besides EGFR, other receptors can be trapped in compression-induced frustrated CCSs, thus leading to sustained signaling in the Erk pathway.

DISCUSSION

Here, we confirmed previous findings showing that cell compression leads to frustrated endocytosis, with an accumulation of long-lived CCSs⁵. Several pieces of evidence point to a predominant role of integrins in CCSs frustration, through local anchoring of the CCSs machinery to the substrate²¹. HeLa cells display numerous frustrated CCSs, also termed clathrin-coated plaques, whose formation depends on local enrichment of the $\alpha\nu\beta5$ integrin⁴. Yet, inhibiting this integrin did not prevent the accumulation of long-lived CCSs in cells experiencing compression. Cell compression most likely results in a dramatic increase in membrane tension that is known to impede CCSs budding^{13,14}. Thus, our data strongly suggest that CCSs frustration, as detected in compressed cells, results from increased membrane tension. We also reported that AP-2 dynamics is perturbed at frustrated CCSs under compression. This may also results from increased membrane tension as this feature is known to modulate CCS components interaction with the plasma membrane¹⁴. An altered dynamics of CCS components is likely to perturb cargo recruitment at CCSs and, indeed, we observed that the TfR is excluded from CCSs under compression. It is not clear why some receptors like the EGFR and the HGFR can still be recruited at compression-induced frustrated CCSs upon stimulation, while the TfR, which is normally constitutively addressed to these structures becomes excluded. This may depends on the different types of endocytic motifs present on receptor cytosolic tails that engage different recognition sites on the AP-2 complex and/or on other CCSs components²². In addition, it is not clear why the dynamics of EGFR is reduced at compression-induced frustrated CCSs. It is possible that the reduced AP-2 dynamics we observed at frustrated CCSs might impact cargo dynamics. In any case, further studies will be required to elucidate if and how altered CCSs components dynamics modulates receptor sorting at CCSs.

We also confirmed previous finding reporting that EGFR becomes activated under pressure, leading to strong Erk activation¹⁰. As reported, this activation seems to depend on metalloprotease-induced HB-EGF shedding leading to paracrine activation of the receptor. However, we observed that EGFR recruitment at CCSs does not depend on the activity of the kinase domain of the receptor. It has long been believed that EGFR autophosphorylation is required for both signaling output and endocytosis of the receptor¹⁶. Yet, some recent studies have suggested that ligand-induced EGFR dimerization is sufficient to induce the accumulation of the receptor at CCSs, without the need for autophosphorylation of the cytosolic tail^{17,23}. Our data clearly support this model.

Finally, we showed that CCSs are required for full Erk activation downstream of the EGFR. These observations are in good agreement with previous reports demonstrating that CCSs can serve as signaling platform for the EGFR^{5,13}. Yet, we previously demonstrated that clathrin-coated plaques can also serve as signaling platform for other receptors⁴. Here, we report that compression-induced CCSs can potentiate HGFR signaling in non-acute stimulation conditions, leading to strong Erk activation even when EGFR kinase activity is inhibited. In these conditions, HGFR also strongly accumulated in CCSs under pressure. It is likely that frustrated CCSs trap the few activated HGFRs and, instead of being internalized, progressively accumulate in these stalled structures. Thus, we propose that cell compression leads to the activation of the EGFR, but also because compression-induced CCSs can trap and potentiate signaling by many other receptors.

METHODS

Cell lines and constructs

HeLa cells (a gift from P. Chavrier, Institut Curie, Paris, France; ATCC CCL-2), genome-edited HeLa cells engineered to expressed an endogenous GFP-tagged or mCherry-tagged µ2 subunit, were grown in DMEM Glutamax supplemented with 10% foetal calf serum at 37°C in 5% CO2. For microscopy, cells were serum-starved for at least 2h before the experiment. All cell lines have been tested for mycoplasma contaminations. mCherry-TfR was a gift from Michael Davidson (Addgene plasmid #55144). GFP-Erk2 was a gift from Dr.Hesso Farhan. EGFR-GFP was a gift from Alexander Sorkin (Addgene plasmid # 32751). pLenti-MetGFP was a gift from David Rimm (Addgene plasmid # 37560).

Plasmids were transfected 24 h after cell plating using either Lipofectamine 3000 according to the manufacturer's instructions or electroporating cells in suspension using AMAXA nucleofector Kit V according to the manufacturer's instructions. Alternatively, linear PEI (MW 25.000 – Polysciences Cat. Nr. 23966) at 1 mg/ml was used to transfect 50 % confluent cells in a 6 well plate according to the following protocol: 2 μ g of DNA were added to 100 μ l of OptiMEM, followed by addition of 4 μ l of PEI, vortex and incubation for 10 minutes at RT prior to add the mix to the cells.

Antibodies and drugs

Rabbit polyclonal antibodies anti tot-ERK1/2 (Cat. Nr. 9102) and P-ERK1/2 (Cat. Nr. 9101) were purchased from Cell Signalling. Mouse monoclonal anti tot-ERK1/2 (Cat. Nr. 13-6200) was purchased from Thermo Fisher. Gefitinib (Cat. Nr. CDS022106) was purchased from Sigma and used at a final concentration of 10 μ M. Cilengitide was purchased from Selleckchem

(Cat. Nr. S7077) and used at a final concentration of 10 μ M. Human recombinant HGF (Cat. Nr. 1404) was purchased from Sigma and used at a final concentration of 100 ng/ml. For HGF experiments, cells were previously serum-starved for at least 2 h and HGF was added to the serum-free medium for 1 h before experiment.

In vitro compression experiments

To investigate the effect of compressive stress on cell behavior, an under-agarose assay was used²⁴. Cells were plated either in 6-well cell culture plates or in glass-bottom dishes (μ-Dish Cat Nr 190301, Ibidi). 24 h hours later, cells were subjected to mechanical stress by using an agarose plug overlaid with the weight necessary to reach a pressure of approximately 1000 Pa. To prepare agarose gels, agar was weighted and dissolved in DMEM Glutamax to a final concentration of 2.4%. The mixture was then casted in an empty dish or well and cooled at room temperature. Agar disks were sterilized under UV light and equilibrated at 37°C before use. For western blots, cells were subjected to compression for 30 minutes prior to cell lysis. For video microscopy, videos were started 30 sec before applying the compressive stress. Compressed cells were then imaged for 30 min. Alternatively, for CCSs dynamics and for FRAP experiments, videos were acquired before and under compression and the videos before compression were compared to the videos under compression.

Western Blots

For Western Blot experiments, cells were lysed in ice cold MAPK buffer (100mM NaCl, 10 nM EDTA, 1% IGEPAL [®] CA-630, 0.1% SDS, 50mM TRIS-HCl pH 7.4) supplemented with protease and phosphatase inhibitors. Protein concentration was measured with Pierce[™]

Coomassie Plus (Bradford) Assay Kit (Cat Nr 1856210) according to the manufacturer's instructions in order to load equal amount of proteins. Antibodies were diluted at 1:1000 in PBS - 0.1% Tween - 5% BSA or 5% non-fat dried milk. For stripping, membranes were incubated in a commercial stripping buffer (Cat. Nr ST010; Gene Bio-Application) according to the manufacturer's instructions. Western-blot quantifications were done in FIJI.

RNA interference

For siRNA depletion, 200 000 cells were plated in 6 well plates. After 24 h, cells were treated with the indicated siRNA (30 nM) using RNAimax (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Protein depletion was maximal after 72 h of siRNA treatment as shown by immunoblotting analysis with specific antibodies. To deplete CHC, α -adaptin or μ 2adaptin, cells were transfected once as described above and then a second time, 48 hours later, with the same siRNAs. In this case, cells were analyzed 96 hours after the first transfection. The following siRNAs were used: µ2-adaptin, 5'-AAGUGGAUGCCUUUCGGGUCA-3'; heavy chain (CHC), 5'GCUGGGAAAACUCUUCAGATT-3'; α-adaptin, 5'-Clathrin AUGGCGGUGGUGUCGGCUCTT-3'; Epidermal growth factor receptor (EGFR) 5'-GAGGAAAUAUGUACUACGA-3' 5'-(EGFR-1) and GCAAAGUGUGUAACGGAAUAGGUAU-3' (EGFR-2); non-targeting siRNAs (siControl), ON-TARGETplus Non-Targeting SMARTpool siRNAs (Dharmacon D-001810-01).

Spinning disk microscopy of live cells

For CCSs dynamics, cells were imaged at 5 s intervals for the indicated time using a spinning disk microscope (Andor) based on a CSU-W1 Yokogawa head mounted on the lateral port of

an inverted IX-83 Olympus microscope equipped with a 60x 1.35NA UPLSAPO objective lens and a laser combiner system, which included 491 and 561 nm 30 mW DPSS lasers (Andor). Images were acquired with a Zyla sCMOS camera (Andor). The system was steered by IQ3 software (Andor). Alternatively, cells were imaged on a Nikon Ti2 Eclipse (Nikon France SAS, Champigny sur Marne, France) inverted microscope equipped with a 60x NA 1.40 Oil objective WD 0.130 and with two cameras: a sCMOS PRIME 95B camera (Photometrics, AZ, USA) and a sCMOS Orca Flash 4.0 (Hamamatsu Photonics France, Massy, France, a dual output laser launch, which included 405, 488, 561 and 642 nm 30 mW lasers, and driven by Metamorph 7 software (MDS Analytical Technologies, Sunnyvale, CA, USA).

For CCS dynamics quantification, the lifetime of CCSs was measured using the TrackMate plugin of ImageJ (Tinevez, 2017^{25}). Tracks below 5 seconds of duration (detected on only 1 frame) were discarded. Measured individual lifetimes were pooled into two groups: the "dynamic" group corresponding to structures with a lifetime below the duration of the movie (5 min) and the "static" group with a lifetime of 5 min. Of note, the relative percentage of dynamic versus static structures depends on the duration of the movie because static structures are only counted once while dynamic structures continuously nucleate and disappear during the movie. For this reason, all quantifications of CCS dynamics represent the relative number of static or dynamic events detectable at the plasma membrane at a given time point. At least 1000 CCSs from at least 5 cells per conditions and per experiments were tracked in 3-5 independent experiments. Data are expressed as mean \pm SD.

Total internal reflection fluorescence microscopy (TIRF) and Fluorescence Recovery After Photobleaching (FRAP)

For total internal reflection fluorescence microscopy (TIRF), HeLa cells transfected with the indicated plasmids were imaged through a 100x 1.49 NA APO TIRF WD 0.13-0.20 oil objective lens on a Nikon Ti2 Eclipse (Nikon France SAS, Champigny sur Marne, France) inverted microscope equipped with two cameras: a sCMOS PRIME 95B camera (Photometrics, AZ, USA) and a sCMOS Orca Flash 4.0 (Hamamatsu Photonics France, Massy, France, a dual output laser launch, which included 405, 488, 561 and 642 nm 30 mW lasers, and driven by Metamorph 7 software (MDS Analytical Technologies, Sunnyvale, CA, USA). A motorized device driven by Metamorph allowed the accurate positioning of the illumination light for evanescent wave excitation.

For TIRF-FRAP experiments, one CCS was manually selected was selected and subjected to 100% laser power (30 mW laser) scan in order to have a bleaching of at least 80% of the fluorescence. One frame was collected before photo-bleaching, and 60 frames were collected after bleaching to analyze fluorescent recovery at the frequency of 1 frame/2 sec. The data were analyzed using the ImageJ FRAP Profiler plugin (McMaster University, Canada) to extract recovery curves and calculate the half-time recovery.

Statistical analyses

Statistical analyses in Fig.1 (panels b, c), Fig.2 (panels b, d, f, h), Fig.3 (panel e), Fig.4 (panel g), Supplementary Fig.3 (panel c) have been performed using One Way Analysis of Variance (ANOVA). Statistical analyses in Fig.3 (panel b), Fig.4 (panel e), Supplementary Fig.4 (panel b), have been performed using two tailed Student's T-test. All data are presented as mean of at

least four independent experiments ± SD. All statistical analyses were performed using SigmaPlot software.

Data availability

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request.

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F.B and D.L.D designed and performed experiments, analysed results and wrote the manuscript. N.E performed experiments. G.M supervised the study, designed experiments and wrote the manuscript.

The authors declare no competing interests. Correspondence and requests for materials should be addressed to <u>guillaume.montagnac@gustaveroussy.fr</u> or to <u>francesco.baschieri@gustaveroussy.fr</u>

Figure legends:

Figure 1. **Cell compression reduces CCSs dynamics. a**, Kymographs showing CCS dynamics in genome-edited HeLa cells expressing endogenous GFP-tagged μ 2-adaptin compressed or not under an agarose plug and treated or not with Cilengitide, as indicated, and imaged by spinning disk microscopy every 5s for 5 min. **b**, **c**, Quantification of the dynamics of CCSs observed as in a (* P<0.001, as compared to 0.1 kPa condition, One Way Analysis of Variance – ANOVA. N=3). **d**, Gallery depicting fluorescence recovery of a single CCS (arrows) after photobleaching in control (upper panels) or compressed (lower panels) cells. Time before or after photobleaching is indicated in seconds. Scale bar: 1µm. **e**, Quantification of fluorescence recovery as in d in control or compressed cells as indicated. All results are expressed as mean ± SD.

Figure 2. **CCSs are required for EGFR-dependent signaling. a**, Western-blot analysis of phospho-Erk (P-Erk) levels in HeLa cells uncompressed (control) or compressed for different time period as indicated (representative image of four independent experiments). Total-Erk and tubulin were used as loading controls. b, Densitometry analysis of bands obtained in Western-blots as in a. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way Analysis of Variance – ANOVA). c, Western-blot analysis of phospho-Erk (P-Erk) levels in HeLa cells compressed or not for 30 min and treated or not with EGFR specific siRNAs as indicated (representative image of four independent experiments). Total-Erk and tubulin were used as loading controls. d, Densitometry analysis of bands obtained in Western-blots as in c. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way analysis of Variance – ANOVA). Total-Erk and tubulin were used as loading controls. d, Densitometry analysis of bands obtained in Western-blots as in c. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way Analysis of Variance –

ANOVA). **e**, Western-blot analysis of phospho-Erk (P-Erk) levels in HeLa cells compressed or not for 30 min and treated or not with Gefitinib as indicated (representative image of four independent experiments). Total-Erk and tubulin were used as loading controls. **f**, Densitometry analysis of bands obtained in Western-blots as in e. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way Analysis of Variance – ANOVA). **g**, Western-blot analysis of phospho-Erk (P-Erk) levels in HeLa cells compressed or not for 30 min and treated or not with AP-2 subunits- or CHC-specific siRNAs as indicated (representative image of four independent experiments). Total-Erk and tubulin were used as loading controls. **h**, Densitometry analysis of bands obtained in Western-blots as in g. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way Analysis of Variance – ANOVA).

Figure 3. EGFR is recruited at CCSs under compression. a, Genome-edited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with a plasmid encoding EGFP-tagged EGFR, seeded on glass, compressed under an agarose plug and imaged by TIRF microscopy every 5s for 30 min. Time after compression is indicated. Higher magnifications of boxed regions are shown. Arrows point to EGFR positive CCSs. Scale bar: 8 μ m. b, Quantification of EGFP-EGFR enrichment at CCSs at the indicated time points after compression in control cells or in cells treated with Batimastat or with Gefitinib, as indicated (* P<0.005, two tailed Student's T-test. N=3; 80 to 100 structures per experiment were analysed.). c, Genome-edited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with a plasmid encoding EGFP-tagged EGFR, seeded on glass, treated with Batimastat, compressed under an agarose plug and imaged by TIRF microscopy every 5s for 30 min. Time after compression is indicated. Scale bar: 1 μ m. d, Western-blot analysis of phospho-Erk (P-Erk) levels in HeLa cells compressed or not and treated or not with Batimastat, as indicated (representative image of four independent experiments). Tubulin was used as a loading control. **e**, Densitometry analysis of bands obtained in Western-blots as in d. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way Analysis of Variance – ANOVA). **f**, Genome-edited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with a plasmid encoding EGFP-tagged EGFR, seeded on glass, treated with Gefitinib, compressed under an agarose plug and imaged by TIRF microscopy every 5s for 30 min. Time after compression is indicated. Scale bar: 1 μ m.

Figure 4. CCSs under compression can serve as signaling platform for different receptors. a-c, Genome-edited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with plasmids encoding EGFP-tagged β 1AR, β -arrestin-2 or HGFR, as indicated, seeded on glass, compressed under an agarose plug for 5 min and imaged by TIRF microscopy. Scale bar: 1.5 µm. **d**, Genome-edited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with a plasmid encoding EGFP-tagged EGFR. Cells were seeded on glass and HGF was added in the culture medium 1h before cells were compressed under an agarose plug and imaged by TIRF microscopy every 5s for 30 min. Time after compression is indicated. Arrows point to HGFR positive CCSs. Scale bar: 1.5 µm. **e**, Quantification of EGFP-HGFR enrichment at CCSs before or 5 min after compression in control cells treated as in d (* P<0.005, two tailed Student's T-test. N=3; 80 to 100 structures per experiment were analysed.). **f**, Western-blot analysis of phospho-Erk (P-Erk) levels in HeLa cells that were incubated in the presence of HGF for 1h before to be compressed or not and treated or not with Gefitinib, as indicated (representative image of three independent experiments). Total Erk was used as a loading control. **g**, Densitometry analysis of bands obtained in Western-blots as in f. Results are expressed as mean ratio of P-Erk/total Erk \pm SD from three independent experiments (* *P*<0.05, One Way Analysis of Variance – ANOVA).

Supplementary figure legends:

Figure S1. **Analysis of cell compression efficiency. a**, Wide-field image of one HeLa cell compressed under and agarose plug. Arrows points to blebs at the plasma membrane. Scale bar: 5μm. **b**, HeLa cells treated with Sir-DNA were imaged by spinning disk microscopy before or after compression under an agarose plug, as indicated. Arrows point to nuclear blebs. Scale bar: 10μm.

Figure S2. Analysis of Erk and Yap behavior under pressure. a, HeLa cells transfected with a plasmid encoding EGFP-tagged Erk were imaged by spinning disk microscopy before (upper panel) or after (lower panel) being compressed under an agarose plug. Scale bar: 10μ m. b, Quantification of EGFP-Erk enrichment in the nucleus at the indicated time points after compression. c, HeLa cells transfected with a plasmid encoding EGFP-tagged Yap were imaged by spinning disk microscopy before (upper panel) or after (lower panel) being compressed under an agarose plug. Scale bar: 10μ m. All results are expressed as mean ± SD.

Figure S3. EGFR activation under compression is serum-independent. a, Genome-edited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with a plasmid encoding EGFP-tagged EGFR, seeded on glass, starved for 2h, compressed under an agarose plug in FCS-free medium and imaged by TIRF microscopy every 5s for 30 min. Time after compression is indicated. Scale bar: 1.5 μ m. b, Western-blot analysis of phospho-Erk (P-Erk) levels in starved HeLa cells compressed or not in FCS-free medium, as indicated (representative image of four independent experiments). Total Erk was used as a loading control. c, Densitometry analysis of bands obtained in Western-blots as in b. Results are expressed as mean

ratio of P-Erk/total Erk \pm SD from four independent experiments (* *P*<0.05, One Way Analysis of Variance – ANOVA). All results are expressed as mean \pm SD.

Figure S4. Alteration of receptor sorting and dynamics under compression. a, Genomeedited HeLa cells expressing endogenous mCherry-tagged μ 2-adaptin were transfected with a plasmid encoding EGFP-tagged TfR, seeded on glass and imaged by TIRF microscopy before (upper panel) or 5 min after (lower panel) compression. Arrows point to TfR positive CCSs. Arrowheads point to TfR-positive, AP-2-negative structures most likely corresponding to endosomes. Scale bar: 2 µm. b, Quantification of EGFP-TfR enrichment at CCSs before or 5 min after compression in cells treated as in a (* P<0.005, two tailed Student's T-test. N=3; 80 to 100 structures per experiment were analysed.). c, Quantification of fluorescence recovery after photobleaching of the EGFP-EGFR fluorescence in individual CCSs in cells stimulated with EGF or compressed under an agarose plug, as indicated. All results are expressed as mean \pm SD.



Figure 1











Figure 4





10 min compression

Figure S1

а



C GFP-Yap - Before compression





Figure S2






Figure S3







Figure S4

DISCUSSION

The results I obtained during my PhD further stress the role of CCS frustration in regulating cell response to mechanical stimuli, as previously proposed by the lab. They also raise a number of questions that would need to be addressed in future studies.

Compression-induced frustrated endocytosis

First, my work demonstrated that cell-compression stalls CCS dynamics and is thus another mechanical parameter that can result in endocytosis frustration, on top of the parameters previously described by the lab such as substrate rigidity and collagen fibers^{176,181}. In the case of substrate rigidity, the underlying mechanism of CCS frustration has been proven to be dependent on a local accumulation of the $\alpha V\beta 5$ integrin. This observation was coherent with reports of a potential role for clathrin plaques in cell adhesion^{181,310}. Indeed, on a stiffer substrate, integrins would engage strongly with the surrounding matrix and this would prevent budding of clathrin-coated vesicles and lead to the formation of flat clathrin plaques instead. However, generation of frustrated CCSs under confinement seems to depend on a different mechanism since inhibiting the $\alpha V\beta 5$ integrin has no effect on AP2 dynamics in this condition. Even though stiffness and confinement seem to be alike because they both are mechanical stresses, the cell response to these modifications appears to be different. How exactly cell compression leads to the formation of frustrated CCSs is not strictly demonstrated but is strongly believed to depend on an increased membrane tension. Indeed, membrane tension is a key regulator of CCS dynamics and budding capacity and compressing cells most likely results in an increased membrane tension¹¹². It is however difficult to measure membrane tension in the assay I used during my PhD because of the lack of accessibility for membrane tether pulling experiments or Atomic force microscopy analyses, two classical means of measuring membrane tension. The recent development of a live-cell fluorescent membrane tension probe (Flipper-TR), could be used in the future to ascertain that membrane tension is indeed increased upon cell

compression³¹¹. It would also be necessary to artificially reduce the membrane tension in compressed cells to determine if this parameter is responsible for CCSs frustration. Although these experiments may prove difficult to perform, one could test hypertonic buffers or membrane incorporation of lipidic vesicles to reduce the tension.

Another point that would need clarification is whether the frustrated structures I observed in compressed HeLa cells are similar in nature to the clathrin-coated plaques these cells naturally develop on rigid substrates. Analyzing clathrin-coated plaques remain challenging, mostly because of the lack of specific marker for plaques. Indeed, plaques present the same molecular pattern as clathrin-coated pits, except for $\alpha V\beta 5$, only seen at plaques but not at dynamic clathrin-coated pits. Yet, frustrated CCSs in compressed cells do not rely on this integrin as I observed in my work. The characteristic of plaques is that they remain flat for a long period of time. Compression-induced frustrated CCSs could also be flat but this remains to be demonstrated. To characterize better these particular structures, the technique which seems the best fitted would be Correlative light-electron microscopy (CLEM). This technique is the combination of an optical microscope, usually a fluorescence microscope, with an electron microscope. CLEM was used in the elegant paper of Sochacki et al. to determine the precise location of endocytic proteins during clathrin-coated vesicle formation⁶¹. Given the peculiar shape of clathrin, CCSs are easily discernable by electron microscopy. However, this technique appears to be a laborious work and very time-consuming and may be particularly difficult to apply to cells under an agarose pad.

Uncoupling between EGFR recruitment to CCSs and EGFR activation

Our results also demonstrate a recruitment of the epidermal growth factor receptor (EGFR) into CCSs under confinement. From here, and from the testing of two drugs already described to inhibit the EGFR signaling directly (as for gefitinib) or indirectly (as for batimastat) but also the experiments in absence of serum, two ideas had emerged. The first experiment on EGFR

was performed on cells grown in a serum-free medium and submitted to mechanical compression showed both a recruitment of these receptors in CCSs, but also a downstream signal linked to it. Alone, this result had led us to think of a ligand-independent EGFR activation. But the experiment using batimastat on starved cells, a drug able to inhibit the shedding of pro-ligands of EGFR at the membrane, inhibited both recruitment of EGFR in CCSs and downstream signaling and thus is in favor of a ligand-dependent activation of the receptor. These results support previous studies about the role of batimastat in EGFR activation²⁷³, but also the mechanical stress-induced activation of sheddases³⁰⁵. However, batimastat inhibits a broad range of metalloproteases, including ADAM 17 and 10, the ones responsible for shedding are able to cleave six different pro-ligands of EGFR²⁷³. To determine which ligand is cleaved by batimastat, it would be interesting to knockdown these pro-ligands with siRNA, specifically HB-EGF and TGF- α since they were both demonstrated to be shedded under mechanical forces³⁰⁵.

The surprising observation we've made was with the use of gefitinib. This specific inhibitor of the EGFR tyrosine kinase activity, blocked the EGFR downstream signaling. This result was expected since its efficacy is well-accepted as an inhibitor of EGFR and gefitinib is a broadly-used molecule in anticancer therapy (especially non-small cell lung cancer (NSCLC)) since 2002³¹². However, the unexpected observation we have made came from the EGFR recruitment at CCSs in gefetinib-treated, confined cells. Whereas the role of EGFR kinase activity in receptor routing for CME has been controversial, it is commonly believed that EGFR phosphorylation is necessary for recruitment to CCSs and signaling^{313,314}. But here, we show an uncoupling between EGFR recruitment to CCSs and its tyrosine kinase activity, supports previous findings of Wang et al. suggesting that receptor dimerization (driven by the ligand) is sufficient to address the EGFR for clathrin-dependent internalization, independently of the activity of the kinase activity for EGFR recruitment to CCSs are already strongly consistent with this model. However, in their study, they also demonstrated that non-ligand induced dimerization of EGFR

is also sufficient to promote receptor internalization³¹⁵. Thus, the experiment previously proposed to uncover the specific ligand for EGFR recruitment to CCSs appears even more essential and would give us the missing piece of the puzzle: whether or not (and if yes, which one) ligand binding is required for EGFR recruitment to CCSs upon compression. Batimastat experiments already point to the requisite of ligand for EGFR relocalization because of its action on ADAMs responsible for ligand shedding, but it could also be an off target effect of another metalloprotease. In these case, non-ligand-induced dimerization could be the key to explain our results.

Redundancy of signaling caused by solid stress

We already reported that a particular subset of frustrated CCSs, clathrin-coated plaques, serve as signaling platforms, not only for the EGFR but virtually for any signaling receptor accumulating at these structures. The exact nature of compression-induced frustrated CCSs is still not clear, but my work suggests that they also participate in signaling as CCS components were required for full Erk activation downstream of EGFR under confinement. In addition, we also observed a role of CCSs as signaling platforms for the HGFR under confinement. In this case, adding the ligand in the medium is necessary but this stresses the fact that, on top of the paracrine activation of EGFR, CCSs' frustration per se is an important factor reinforcing cell signaling in cells under pressure. It is thus very likely that compression-induced CCSs frustration is part of the cell response to this harmful condition and further functional studies will be required if this helps the cell to better cope with compression. Given the plethora of signaling receptors recruited at CCSs, frustrated CCSs could potentially impact many different signaling pathways. Of note, the exclusion of the transferrin receptor from CCSs observed under pressure (see below) is interesting and suggests that some other receptors may as well be excluded, some of which may actually need CCSs in order to fully signal. Thus, one can imagine that frustrated CCSs may fine-tune signaling pathways to provide and adapt answers to mechanical perturbations.

Differential receptor recruitment under confinement

Regarding other receptors recruitment into CCSs under confinement, we made an unpredictable observation. As said before, EGFR is recruited to CCSs when the cells are subjected to compression. We did not observe a similar phenotype for β 1-adrenergic G-protein coupled receptor (GPCR), GPCR clathrin adaptor β -arrestin 2, and hepatocyte growth factor receptor (HGFR), receptors or adaptors usually all recruited to CCSs upon their activation. This simply reflects the necessity for ligands and that only EGFR ligands are specifically released in compressed cells. However, by looking at the transferrin receptor (TfR), which is usually constitutively recruited at CCSs independently of ligand binding and considered as the major marker of CME, we revealed its exclusion from CCSs under compression. This observation reignites the debate about specialization of CCSs for specific cargoes. Two opposite models have been proposed; the first one supports the idea of subpopulations of CCSs specialized in the entry of one specific cargo while other receptors become excluded^{316,317}, whereas the alternative one, commonly accepted, implies the internalizing of a mixture of cargoes by all CCSs and differential sorting only after their uptake¹⁵⁴. One study have focused on the specificity of CCSs for TfR and uncover the role of TTP, a SH3-containing protein as an important regulator of TfR uptake³¹⁸. The authors also observed an exclusion of TTP from some CCSs, but the underlying mechanism remains obscure although they propose a role for kinase-mediated phosphorylation of TTP in this exclusion. In this study, TPP has been identified of a specific partner in TfR endocytosis, and considering the differences between the TfR and the EGFR for internalization, other cargo-specific adaptors could participate to the differential recruitment we observed.

The main difference between TfR and EGFR is their endocytic signals. TfR is recognized by the μ 2 subunit of AP2 through its YXXØ motif in the cytoplasmic domain. As for EGF receptor, its internalization relies on ubiquitin signals recognized by Eps15 and epsins strongly bound to AP2 appendages. In addition, the EGF receptor also presents a classical tyrosine-based motif (Y⁹⁷⁴RAL) and a dileucine motif (Leu¹⁰¹⁰/Leu¹⁰¹¹) both able to bind the AP2 complex although none of them are necessary for clathrin-mediated endocytosis³¹⁹⁻³²¹.

Distinct requirement of recognition sites of AP2 or the involvement of additional partners could explain why TfR is excluded from CCSs in favor of EGFR under compression. Also, since EGFR also contain classical internalizing motifs, mutating regions of ubiquitination to see if this receptor is still recruited to CCSs under confinement could be interesting to test, and competition with TfR would be a conceivable explanation. To test the hypothesis of partners, mutant forms of either Eps15 or epsins on their ubiquitin-interacting motifs or on the AP2-appendages recognition domain for these two proteins could give us further understanding of the underlying mechanism. It would be also interesting to test whether the kinase activity of EGFR is responsible for the exclusion of TfR from CCSs, simply by observing by microscopy the localization of TfR in compressed cells previously treated with gefitinib (the specific inhibitor of EGFR kinase activity)

Dynamics of receptors under pressure

It is extensively believed that mechanical forces are sensed and transformed into biochemical signals by receptors at the plasma membrane. However, since the PM is the first element in contact with the outside, mechanical perturbations are expected to lead to changes in the membrane composition or dynamics of its components. We first tested the effects of compression on the dynamics of CCSs themselves as well as on the AP2 complex, a key structural components of CCSs. In order to do so, we perform a fluorescence recovery after photobleaching (FRAP) experiment in our genome-edited HeLa cells expressing eGFP-tagged, endogenous µ2-adaptin. Compared to the control condition, in which cells are not subjected to any pressure, the fluorescence recovery is significantly reduced in confined cells. It is no clear why cell compression would reduce the dynamics of AP2 at CCSs. We can postulate that an AP2 pool usually diffusing at the plasma membrane would be slowed down because of an increased membrane tension⁶⁴. Another, non-exclusive possibility is that the turnover of AP2 is delayed, possibly because it is stuck at CCSs. In any case, this reduced AP2 dynamics mirrors the reduced EGFR dynamics we observed at CCSs under confinement. It is tempting to

speculate about a functional link between these two observations. Given the central role of AP2 in organizing CCSs, a reduction of its turnover probably would reduce the turnover of receptors relying on it to be recruited at CCSs. EGFR has been reported to be still recruited at CCSs in the absence of AP2³²². However, more recent studies suggested a requirement for AP2 in EGFR recruitment in physiological conditions^{323,324}. In addition, we only tested the dynamics of AP2 under confinement here. Other adaptors or accessory proteins may also have a reduced turnover under pressure. Further studies will be required to understand how cell compression affects the dynamics of individual CCSs components and cargoes. An exciting possibility is that compression actually challenges the whole plasma membrane dynamics and this would be a key to understand how PM-associated structures and proteins are affected. Along this line, I observed in preliminary data a reduction of the dynamics of a fluorescent WGA (wheat germ agglutinin), which binds to glycolipids and glycoproteins at the PM, under compression. Thus, membrane fluidity could be affected under compression and this would be the main driver of the perturbations observed in these conditions.

All together, the results obtained in this PhD project revealed an active participation of CCSs in cell response to confinement. We were able to observe frustrated endocytosis caused by confinement, as it was previously demonstrate for stiffness. Regarding the role of these structures on signaling, they indeed stimulate the MAPK/Erk pathway by retaining EGFR at the cell surface, where they keep signaling. Moreover, and since the activity of EGFR upon compression seems to be highly specific, we uncover a very possible mechanism responsible for this activation implicating EGFR pro-ligand shedding, whereas further studies will be required to assess this prior assumption.

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ANNEXE : RESUME DE LA THESE EN FRANÇAIS

L'endocytose dépendante de la clathrine (EDC) est la principale voie d'internalisation des récepteurs de surface et de leurs ligands. L'internalisation se fait suite à l'invagination de la membrane plasmique vers l'intérieur de la cellule suite à la formation, dans un premier temps, de puits recouverts de clathrine (PRCs) qui bourgeonnent ensuite en vésicules recouvertes de clathrine dans le cytosol. L'EDC est un processus très dynamique qui a lieu en l'espace de 30 sec-1mn. Elle est impliquée dans de multiples fonctions et permet ainsi à la cellule de réguler l'expression de ses protéines en surface, de répondre aux signaux de prolifération ou migration envoyés par l'environnement immédiat via l'activation de voies de signalisation spécifiques ou encore de réguler le renouvellement des composants de la membrane plasmique. De par son importance, des dérégulations de l'endocytose dépendante de la clathrine ont déjà été observées dans les cancers. Ces modifications peuvent impliquer directement l'EDC en modifiant ses composants ou indirectement lors d'altérations de récepteurs régulés par celle-ci. La progression tumorale est elle-même régulée par de multiples facteurs, notamment l'accumulation de mutations qui ont des conséquences sur les cellules cancéreuses elles-mêmes ou bien sur l'environnement immédiat, formant ainsi la « niche tumorale ». Ces changements agissent réciproquement sur la progression tumorale afin de l'amplifier. Lors de la croissance tumorale, les cellules cancéreuses recrutent des fibroblastes qui vont participer au remodelage et à l'augmentation de la rigidité autour de la tumeur. La rigidité de la matrice extracellulaire est détectée par les cellules ce qui envoie des signaux déclencheurs de prolifération et de migration en conséquence. Cette détection passe essentiellement par les intégrines à la surface membranaire qui vont s'agréger et induire des cascades de signalisation impliquées dans ces réponses. Ces intégrines peuvent se regrouper dans deux types de structures, les adhésions focales et les structures recouvertes de clathrine. En ce qui concerne ces dernières, il a été démontré précédemment que la rigidité du substrat augmente sa force d'interaction avec les intégrines, et empêche ainsi l'internalisation des vésicules recouvertes de clathrine, on parle alors d' « endocytose frustrée ». Cette rétention des structures recouvertes de clathrine à la surface provoque une signalisation soutenue en surface au lieu de l'arrêter par dégradation ultérieure des récepteurs dans les lysosomes. Le laboratoire a démontré que les structures de clathrine frustrées capturent ainsi différent récepteurs conduisant à une signalisation accrue dans la voie de la MAP Kinase Erk. Mon projet de thèse repose sur ces observations en s'intéressant plus particulièrement au rôle d'une autre modification induite par la croissance tumorale, le confinement. En effet, en se multipliant de manière incontrôlée dans un environnement spatialement restreint, les cellules tumorales se retrouvent soumises à des forces de compression. Les résultats mis en évidence au cours de ma thèse ont montré que le confinement provoque, comme la rigidité, une frustration des structures de clathrine qui ne sont donc plus capables de soutenir l'endocytose des récepteurs. De plus, la compression cellulaire induit le clivage d'un pro-ligand de l'EGFR, le HB-EGF, ce qui conduit à l'activation paracrine de l'EGFR et à l'activation de la voie Erk. En effet, l'absence de facteurs de croissance dans le milieu ainsi que l'inhibition de ce clivage démontrent la nécessité de la mise en place de ce mécanisme. En résumé, le confinement induit le clivage du pro-ligand HB-EGF, qui à son tour va activer le récepteur à l'EGF. En parallèle, l'endocytose est ralentie et provoque une signalisation accrue à la membrane. Ces deux évènements coopèrent pour mener à une très forte activation de la voie Erk. Ces résultats mettent en évidence le lien entre un paramètre physique de l'environnement tumoral et une voie de signalisation connue pour contrôler la croissance tumorale.



Titre : Rôle du confinement sur l'endocytose dépendante de la clathrine

Mots clés : Endocytose, clathrine, confinement, EGFR, HB-EGF, Erk

Résumé : L'endocytose dépendante de la clathrine (EDC) est la principale voie d'internalisation des récepteurs de surface et leurs ligands. La membrane plasmique s'invagine et forme des puits recouverts de clathrine (PRCs) qui bourgeonnent ensuite en vésicules recouvertes de clathrine dans le cytosol, le tout en 30 sec-1mn. Lors de la progression tumorale, le microenvironnement est remodelé et la rigidité autour de la tumeur augmente, formant ainsi la « niche tumorale ». La rigidité est détectée principalement par les intégrines en surface qui envoient alors des signaux déclencheurs de prolifération et de migration. Ces intégrines peuvent se regrouper dans les structures recouvertes de clathrine (SRCs). La rigidité du substrat augmente sa force d'interaction avec les intégrines et empêche ainsi l'internalisation des SRCs, on parle alors d'« endocytose frustrée ». Cette rétention des SRCs contenant de multiples récepteurs provoque une signalisation soutenue en surface, notamment de la voie de la MAP Kinase Erk, au lieu de l'arrêter

par dégradation ultérieure dans les lysosomes. Mon projet de thèse repose sur ces observations et plus particulièrement sur le rôle d'une autre modification induite par la croissance tumorale, le En effet. confinement. en se multipliant excessivement dans un environnement spatialement restreint, les cellules tumorales se retrouvent soumises à des forces de compression. Mes résultats ont montré que le confinement provoque, comme la rigidité, la frustration des structures de clathrine, dès lors incapables d'internaliser des récepteurs, menant à une signalisation accrue à la membrane. De plus, le confinement induit le clivage d'un pro-ligand de l'EGFR, le HB-EGF, conduisant à l'activation paracrine de l'EGFR et à l'activation de la voie Erk. En effet, l'absence de facteurs de croissance dans le milieu ainsi que l'inhibition de ce clivage démontrent la nécessité de la mise en place de ce mécanisme. Ces deux évènements coopèrent pour mener à une forte activation de la voie Erk.

Title : Role of confinement on clathrin-mediated endocytosis

Keywords : Endocytosis, clathrin, confinement, EGFR, HB-EGF, Erk

Abstract : Clathrin-mediated endocytosis (CME) is the major endocytic route in eukaryotic cells. The plasma membrane progressively invaginates from clathrin-coated pits (CCPs) to the formation of a clathrin-coated vesicle budding off into the cytosol in around 30 seconds. CME enables cells to sample the cell's environment, to control the activation of signaling pathways and to turn over membrane components and cargoes. During tumor progression, the microenvironment is remodeled and its rigidity increases, thus forming the tumor niche. This stiffness is sensed by integrins which can cluster in clathrincoated structures (CCSs) and initiate signals for proliferation and migration. High stiffness was shown to strengthen the interaction between integrins and the substrate, hence preventing CME, and this is referred to as "frustrated endocytosis" resulting in a sustained signaling instead of down-regulation of the signal by

the endo-lysosomal pathway. My PhD project relied on these previous findings, with a particular focus on another mechanical alteration observed in tumors, confinement. Indeed, the uncontrolled proliferation of cancer cells in a spatially restricted area increases compressive forces. My results indicate that confinement leads to frustrated endocytosis and hence to sustained signaling at the plasma membrane. In addition, compression promotes HB-EGF shedding at the surface, and the resulting EGF product activates the EGFR in a paracrine manner and the Erk signaling pathway. Indeed, both the absence of EGFR ligands in the medium and the inhibition of the shedding demonstrate the necessity of this mechanism for EGFR activation. Together, these events cooperate to strongly activate the Erk pathway known to govern tumor growth.