



HAL
open science

Adhesive Clathrin Structures Support 3D Haptotaxis Through Local Force Transmission

Enzo Bresteau

► **To cite this version:**

Enzo Bresteau. Adhesive Clathrin Structures Support 3D Haptotaxis Through Local Force Transmission. Cellular Biology. Université Paris Saclay (COMUE), 2019. English. NNT : 2019SACLS546 . tel-03478533

HAL Id: tel-03478533

<https://theses.hal.science/tel-03478533>

Submitted on 14 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

ADHESIVE CLATHRIN-COATED STRUCTURES SUPPORTS 3D HAPTOTAXIS THROUGH LOCAL FORCE TRANSMISSION

Thèse de doctorat de l'Université Paris-Saclay
préparée à Gustave Roussy

École doctorale n°582 : cancérologie : biologie - médecine - santé
(CBMS)

Thèse présentée et soutenue à Villejuif, le 13 Décembre 2019, par

Enzo Bresteau

Composition du Jury :

Christian POÛS Professeur des universités, Université Paris Sud	Président
Nathalie SAUVONET Directeur de recherche, Institut Pasteur	Rapporteur
Pablo VARGAS Chargé de recherche, Institut Curie	Rapporteur
Patrick CASWELL Senior Lecturer, Université de Manchester	Examineur
Guillaume MONTAGNAC Directeur de recherche, Gustave Roussy	Directeur de thèse

GUSTAVE ROUSSY INSTITUTE
- PARIS SACLAY UNIVERSITY -

DOCTORAL THESIS

**ADHESIVE CLATHRIN-COATED
STRUCTURES SUPPORTS 3D HAPTOTAXIS
THROUGH LOCAL FORCE TRANSMISSION**

By

ENZO BRESTEAU

Under the supervision of

GUILLAUME MONTAGNAC

13th DECEMBER 2019

JURY

Christian POÛS - University of Paris Sud

Nathalie SAUVONET - Pasteur Institute

Pablo VARGAS - Curie Institute

Patrick CASWELL - University of Manchester

Par amour du goût

AKNOWLEDGMENTS

Je tiens d'abord à remercier les membres de mon jury pour avoir pris le temps de juger mon manuscrit et la présentation de mes travaux de thèse. J'aimerais ensuite remercier Guillaume pour m'avoir pris comme étudiant en thèse. Travailler avec toi est à la fois agréable et très enrichissant, je mesure la chance que j'ai eu. J'aimerais aussi remercier Nadia et Francesco pour le temps phénoménal qu'ils ont pris sur leurs propres projets pour m'aider à faire le mien. Sans leur aide, ce manuscrit aurait été largement différent. Je remercie aussi tous les gens avec qui j'ai travaillé et qui ont participé indirectement à ce manuscrit. Je dois aussi remercier Nathalie pour ne m'avoir jamais crié dessus. Enfin et surtout, je remercie mes parents pour leur amour et leur soutien constant, il est le plus important.

TABLE OF CONTENTS

AKNOWLEDGMENTS	3
TABLE OF CONTENTS	4
ABBREVIATIONS	8
INTRODUCTION	11
I - CELL MIGRATION	11
I.1 - WHERE AND WHEN CELLS MIGRATE	11
I.1.1 - Cell migration in development and homeostasis	11
I.1.2 - Cell migration in pathology	12
I.2 - DIFFERENT MODES OF MIGRATION	14
I.2.1 - Mesenchymal versus amoeboid migration	14
I.2.1.1 - Mesenchymal-like migration	14
I.2.1.2 - Amoeboid migration	15
I.2.2 - Collective cell migration	16
I.3 - MECHANISMS OF CELL LOCOMOTION	16
I.3.1 - The actin cytoskeleton: the engine of cell migration	18
I.3.1.1 - Actin and actin regulators	18
I.3.1.2 - The lamellipodia	20
I.3.1.3 - Other actin structures in cell migration	22
I.3.2 - Adhesion structures	26
I.3.2.1 - ECM receptors	26
I.3.2.2 - Focal adhesions	29
I.3.2.3 - Adhesive clathrin-coated structures	34
I.3.3 - Orientation of cell migration	34
I.3.3.1 - General mechanisms of chemotaxis and haptotaxis	35
I.3.3.2 - Regulation of directed migration	35
I.4 - THE EXTRACELLULAR MATRIX	38
I.4.1 - Composition and structure	39
I.4.1.1 - Glycosaminoglycans and proteoglycans	39
I.4.1.2 - Fibrillar proteins	40
I.4.2 - Collagen type I	40
I.4.2.1 - Collagen structure and fibrillogenesis	41
I.4.2.2 - Collagen binding proteins	42
I.4.3 - Growth factors and the ECM	43
I.4.3.1 - Growth factors binding the ECM	44

I.4.3.2 - Growth factor gradients <i>in vivo</i>	44
I.4.4 - 2D versus 3D ECM and consequences for cell migration	45
I.4.4.1 - Matrix degradation.....	46
I.4.4.2 - Cell adhesion in 3D	46
II - CLATHRIN-MEDIATED ENDOCYTOSIS	48
II.1 - CLATHRIN-COATED STRUCTURES.....	48
II.1.1 - Main actors of CCSs.....	49
II.1.1.1 - Adaptor protein 2.....	50
II.1.1.2 - Clathrin.....	51
II.1.1.3 - Accessory proteins	52
II.1.2 - Life cycle of CCSs	53
II.1.2.1 - CCS nucleation.....	53
II.1.2.2 - CCS maturation	54
II.1.2.3 - Vesicle fission	56
II.1.2.4 - Coat disassembly	57
II.2 - FUNCTIONS OF CLATHRIN-COATED STRUCTURES	58
II.2.1 - Canonical consequences of CCSs as endocytic structures	58
II.2.1.1 - Regulation of plasma membrane composition	59
II.2.1.2 - Regulation of nutrients acquisition.....	59
II.2.1.3 - Regulation of signaling pathways	60
II.2.1.4 - CME and cell migration	61
II.2.2 - Non-canonical functions of CCSs	62
II.2.2.1 - Role of CCSs as signaling platforms.....	62
II.2.2.2 - Role of CCSs in adhesion.....	64
III - THE EPIDERMAL GROWTH FACTOR RECEPTOR	66
III.1 - EGF AND EGFRs FAMILY	66
III.1.1 - The receptor tyrosine kinase family.....	66
III.1.2 - The EGFR family.....	67
III.1.2.1 - EGFR family members	68
III.1.2.2 - Ligands of the EGFR family	69
III.1.2.3 - The epidermal growth factor.....	69
III.2 - PHYSIOLOGY OF THE EGFR	69
III.2.1 - EGFR activation.....	70
III.2.1.1 - EGFR dimerization	70
III.2.1.2 - EGFR transphosphorylation.....	71
III.2.1.3 - EGFR inhibitors	71
III.2.2 - EGFR signaling.....	71
III.2.3 - EGFR endocytosis.....	74

III.2.3.1 - Mechanisms of EGFR uptake	74
III.2.3.2 - EGFR intracellular trafficking	76
III.3 - EGFR IN CELL MIGRATION	77
III.3.1 - EGFR in cell motility	77
III.3.2 - EGFR in chemotaxis	78
III.3.3 - EGFR and the ECM	80
AIM OF THE THESIS	82
RESULTS	84
Abstract	85
Main Text	86
INTRODUCTION	86
RESULTS	87
<i>Production and characterization of ligand-decorated collagen fibers</i>	87
<i>Exacerbated accumulation of TCALs along ligand-decorated fibers</i>	88
<i>Local TCALs accumulation regulates local forces applied on collagen fibers</i>	90
<i>Local TCALs accumulation orients cell migration in 3D</i>	91
METHODS	94
References	103
Acknowledgment	105
Figure legends	106
Supplementary figure legends	110
Figures	113
Figure 1. TCALs preferentially accumulate along ligands-decorated fibers.	113
Figure 2. Increased nucleation rate of TCALs on EGF-decorated fibers.	114
Figure 3. TCALs allow cells to preferentially remodel ligands-decorated fibers.	115
Figure 4. TCALs regulate 3D haptotaxis towards EGF-decorated fibers.	116
Supplementary figures	117
Supplementary Figure 1. Characterization of ligands association with collagen networks.	117
Supplementary Figure 2. EGF on collagen fibers is functional.	118
Supplementary Figure 3. Gefitinib treatment does not prevent EGFR recruitment at CCSs.	119
Supplementary Figure 4. β 1-integrin and vinculin equally distribute between EGF-decorate and non-decorated fibers.	120
Supplementary Figure 5. Characterization of collagen fibers remodelling.	121
DISCUSSION	122
1. - At least some CCS ligands can bind to collagen fibers	122
2. - Collagen-bound EGF is active and can be internalized	123
3. - Accumulation of TCALs along collagen fibers decorated with CCS ligands	124
4. - Cells protrude more on EGF-coated collagen fibers	126

5. - Cells pulls more on CCS ligands-decorated collagen fibers	126
6. - In 3D, cells migrate towards CCS ligands-decorated collagen networks	128
7. - Model and conclusion	128
ANNEX	130
Tubular clathrin/AP-2 lattices pinch collagen fibers to support 3D cell migration.....	130
REFERENCES.....	139

ABBREVIATIONS

ADAMS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ADF	Actin Depolymerizing Factor
ADP	Adenosine DiPhosphate
AP180	Adaptor Protein 180
AP-2	Adaptor Protein 2
ARH	Autosomal Aecessive Hypercholesterolemia
Arp2/3	Actin-Related Proteins 2/3
ATP	Adenosine TriPhosphate
BAD	Bcl-2-Associated Death promoter
BAR	Bin-Amphiphysin-Rvs
BMP	Bone Morphogenetic Protein
BSE	Bundle Signaling Element
CCP	Clathrin-Coated Pits
Cbl	Casitas b-cell lymphoma
CCR	Chemokine Receptor
CCS	Clathrin-Coated Structure
Cdc42	Cell division cycle 42
CHC	Clathrin Heavy Chain
CALM	Clathrin Assembly Lymphoid Myeloid leukemia
CLC	Clathrin Light Chain
CME	Clathrin Mediated Endocytosis
Dab2	Disabled Homolog 2
DAG	Diglyceride
DDR	Discoidin Domain Receptor
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extra Cellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EMT	Epithelial–mesenchymal transition
Eps15	EGFR pathway substrate 15
ER	Endoplasmic Reticulum
ErbB	avian Erythroblastosis oncogene B
Erk	Extracellular regulated kinase
ERM	Ezrin Radixin Moesin

FA	Focal Adhesion
FAK	Focal Adhesion Kinase
FBS	Foetal Bovine Serum
FCHo	Fer/Cip4 Homology domain-only proteins
FGF	Fibroblast Growth Factor
FRAP	Fluorescence Recovery After Photobleaching
GAG	GlycosAminoGlycans
GFP	Green Fluorescent Protein
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
GTP	Guanosine TriPhosphate
GEF	Guanine nucleotide Exchange Factor
HB-EGF	Heparin-Binding EGF-like Growth Factor
HER	Human Epidermal growth factor Receptor
HGF	Hepatocyte Growth Factor
HS	Heparan Sulfate
HSC70	Heat Shock Cognate 70
HSPG	Heparan Sulfate Proteoglycans
IGF	Insulin-like Growth Factor
IP3	Inositol trisPhosphate
LDL	Low Density Lipoprotein
LDLR	Low Density Lipoprotein Eceptor
MAPK	Mitogen Activated Protein Kinase
MMP	Matrix Metalloprotease
mRNA	Messenger Ribonucleic acid
MTOC	MicroTubule Organizing Center
mTOR	mechanistic Target Of Rapamycin
NPF	Nucleation-Promoting Factor
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
PG	ProteoGlycan
PH	Pleckstrin Homology
PTB	PhosphoTyrosine Binding
PI3K	PhosphoInositide 3-Kinase
PIP2	Phosphatidylinositol-(4,5)- bisPhosphate
PIP3	Phosphatidylinositol-(3,4,5)-trisPhosphate
PKC	Protein Kinase C
PLC	PhosphoLipase C

PVR	Polio Virus Receptor
PRD	Proline Rich Domain
PTB	PhosphoTyrosine Binding
PTEN	Phosphatase and TENsin homolog
Rac	Ras-related C3 botulinum toxin substrate 1
Raf	Rapidly Accelerated Fibrosarcoma
Ras	Rat sarcoma
Rho	Ras homolog
ROCK	Rho-associated protein kinase
RLC	Regulatory Light Chain
RTK	Receptor Tyrosine Kinase
siRNA	small interfering RiboNucleic Acid
Sos	Son of sevenless
SH2	Src Homology-2
STAT	Signal Transducer and Activator of Transcription
TCAL	Tubular Clathrin/AP-2 Lattices
TfR	Transferrin Receptor
TGF	Transforming Growth Factor
TKD	Tyrosine Kinase Domain
TIRF	Total Internal Reflection Fluorescence
TKI	Tyrosine Kinase Inhibitor
VASP	VAsodilator-Stimulated Phosphoprotein
VCA	Verprolin momology/Cofilin/Acidic
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoprotein
WASH	Wiskott-Aldrich Syndrom protein and Scar Homolog
WASP	Wiskott-Aldrich Syndrom Protein
WAVE	WASP family Verprolin Homologous Protein
WH2	WASP Homology domain 2
WHAMM	WASP homologue associated with actin, membranes and microtubules

INTRODUCTION

I - CELL MIGRATION

In physics, a movement is the displacement of a body relative to a fixed point in space called a reference frame. Movement requires energy, which can be from external or internal origins. This energy also needs to be transformed into a work force that produces movement. There are several ways to transform a source of energy into a work force. Cell migration, or cell motility, refers to the ability of a cell to actively move relative to its environment. Its energy comes from internal metabolic origins as active migration is by definition cell-autonomous. It is most of the time translated into a work force through dynamic remodeling of the actin cytoskeleton. Finally, these forces need to be applied on the cell environment in order to produce movement. Thus, cell motility is heavily dependent on its environment and, in some cases, the environment can dictate the basic parameters of motion.

I.1 - WHERE AND WHEN CELLS MIGRATE

Cell migration is a fundamental process during the development and homeostasis of multicellular organisms. It also occurs in unicellular organisms as for example amoeba migrate as single cells to colonize new environments. In the case of more complex, multicellular organisms, cell migration occurs during the whole life of every individual. It is especially important during embryonic development, but it is also needed for maintaining physiological functions and, at last, it also plays an important role in several pathology.

I.1.1 - Cell migration in development and homeostasis

During development, cells from different lineages migrate through the embryo to reach their final destination. There are multiple well-studied examples of cell migration during development: the neural crest migration, the gastrulation movements, primordial germ cells

migration or angiogenesis. Defects in cell migration during embryogenesis leads to severe consequences ranging from embryo malformation to lethality (Aman and Piotrowski 2010).

Cell migration is also at the center of the immune response. Monocytes and granulocytes migrate chase pathogens in the organisms through migration. Dendritic cells collect antigens before migrating to lymphoid tissues to trigger T lymphocytes activation. Once activated, T lymphocytes migrate towards peripheral tissues, for example to eliminate infected cells (Parkin and Cohen 2001).

Another striking example of the importance of cell migration in homeostasis is during wound healing. After the initial injury, leukocytes migrate towards the damaged tissue to clean it from dead cells and pathogens. Then, an angiogenic process driven by the directed migration of endothelial cells allows to form new blood vessels. Finally, epithelial cells migrate collectively to close the wound and reseal the epithelium (Gurtner et al. 2008).

I.1.2 - Cell migration in pathology

Deregulated cell migration can play a role in many diseases, through impacting the biological processes cited above and that rely on cell migration. In addition, cell migration plays a central role in cancers. Most solid cancers are from epithelial origins. During the development of the disease, tumoral cells acquire the capacity to migrate, leading to cancer cells leaving the primary tumor and disseminating in the organism to form distant metastases (Figure 1). This is of central importance in the disease as metastases development is widely accepted to be the main cause of cancer-associated mortality. To undergo metastasis dissemination, cancer cells are believed to reactivate a cell migration program through the epithelial to mesenchymal transition (EMT), a process initially describe during embryogenesis (Kalluri and Weinberg 2009). Once able to move, tumor cells degrade the basal membrane separating the epithelium from the stroma in order to leave the primary tumor and migrate through the stroma in order to reach the circulatory system. Circulating cancer cells can then extravasate from blood or lymphatic vessels and migrate until they start to proliferate again to form a metastasis (Lambert, Pattabiraman, and Weinberg 2017; Figure 1). In addition tumor cells also hijack angiogenesis, attracting and directing endothelial cell migration in order to support their own growth (Ide 1939; Greenblatt and Shuvi 1968)

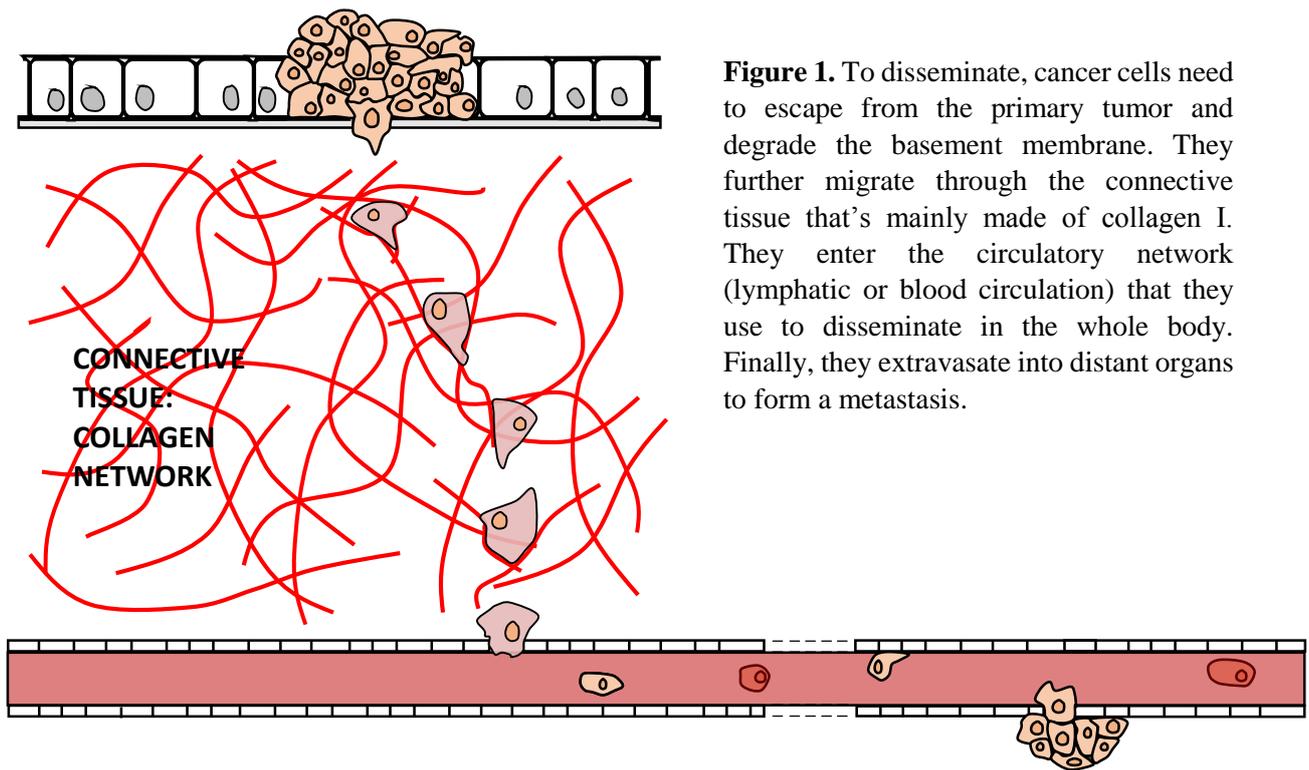


Figure 1. To disseminate, cancer cells need to escape from the primary tumor and degrade the basement membrane. They further migrate through the connective tissue that's mainly made of collagen I. They enter the circulatory network (lymphatic or blood circulation) that they use to disseminate in the whole body. Finally, they extravasate into distant organs to form a metastasis.

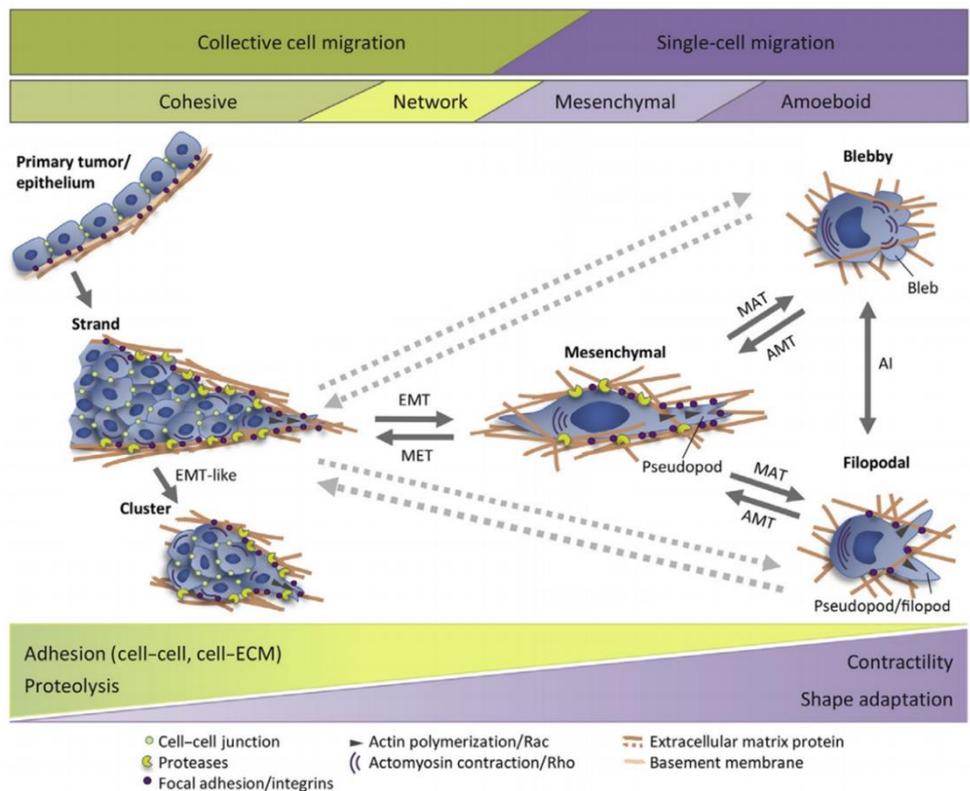


Figure 2. Plasticity of cancer cell migration. Cancer cells can change their migration strategies to fit their environment. Over time, they can switch between single or collective migration, mesenchymal or amoeboid mode of migration. Sub-strategies exist within these main categories. (From Boekhorst et al, 2016)

I.2 - DIFFERENT MODES OF MIGRATION

In order to migrate, cells can adopt different strategies. While it is generally assumed that certain cell types move in a given manner, it is more and more clear that some cells, and especially cancer cells, can switch between different modes of migration depending on the conditions of the microenvironment as well as on some internal properties (Figure 2).

I.2.1 - Mesenchymal versus amoeboid migration

When considering single-cell migration, two main modes of locomotion can be discriminated: mesenchymal-like or amoeboid-like migration. Even if they are here presented distinctly, they must be considered as the two extremes of a continuum. Indeed, although cells often show characteristics predominantly evoking a defined mode of migration, they usually express some characteristics of both types.

I.2.1.1 - Mesenchymal-like migration

Mesenchymal migration is defined by an analogy to the mode of migration of mesenchymal cells, i.e. mostly fibroblasts. This is characterized by cells adopting an elongated, polarized shape with a protruding leading edge at the front and a trailing edge at the back of the cell. This type of migration is usually observed in relatively slow processes as mesenchymal cell velocity typically range around $1 \mu\text{m}\cdot\text{min}^{-1}$ in 3D collagen matrix, which is quite slow as compared to the amoeboid migration of certain cells (Niggemann et al. 1997). The mesenchymal strategy is used by most of the migrating cells during embryogenesis (Kurosaka and Kashina 2008), by fibroblasts and keratinocytes in wound healing (Pilcher et al. 1997; Schmidt et al. 1993), by endothelial cells during angiogenesis (Rousseau et al. 2000), by fibroblasts to maintain a healthy stroma and finally by most carcinoma, sarcoma or melanoma cells (Friedl and Wolf 2003). During mesenchymal migration, actin polymerization at the leading edge pushes onto the membrane, thus forming a protrusion. This protrusion is stabilized by newly formed adhesions which strongly link the extracellular matrix to the actin network. This leading edge also secretes proteases in order to degrade the extracellular matrix (ECM), allowing to excavate passageways in the dense matrix. Acto-myosin-regulated cell contraction allows the cell to pull itself forward while the trailing edge retracts following adhesion structures disassembly. Repetition of this cycle of protrusion, adhesion to the substrate,

disassembly of adhesions at the rear and retraction is the very core of mesenchymal cell migration (Lauffenburger and Horwitz 1996).

I.2.1.2 - Amoeboid migration

Amoeboid migration is famously used during the immune response and so is also observed in immune cells-associated cancers such as lymphoma and leukemia. Even if amoeboid migration mechanisms remain largely unknown, general models have been drawn during the last decades.

Amoeboid cells usually exhibit a roundish form that is completely different from the elongated shape of mesenchymal cells. Amoeboid-like migration strategy is based on cells changing shape to squeeze through its environment. Consequently, amoeboid migration is usually regarded as independent of matrix remodeling (Wolf et al. 2003) even though this vision has been challenged (Orgaz et al. 2014). Amoeboid movement is based on actin contraction at the rear of the cell, rather than actin polymerization at the front as it is the case for mesenchymal migration. Strong actomyosin cortex contractions at the rear compress the cytoplasm, generating a flux that consequently pushes the plasma membrane forward by forming large membrane blebs (Yumura, Mori, and Fukui 1984). Bleb formation is due to either local breakage in the actomyosin cortex, or local detachment of the cortex from the plasma membrane (Keller and Egli 1998), as a direct consequence of cytoplasmic pressure. Upon bleb expansion, the actin cortex reforms inside the bleb leading either to its retraction (not useful for migration) or to its stabilization in order to initiate the translocating process. Bleb formation and stabilization induce a center of mass displacement that, together with nucleus translocation resulting from contractions at the rear, lead to the final movement.

Opposite to mesenchymal-like migrating cells, the amoeboid-like migrating cells make no strong interaction with its environment and is based on light contacts rather than proper, strong adhesive structures.

Because it uses cytoplasm fluxes, cell velocity is not limited by actin polymerization speed. This migration mode is used for fast processes such as the immune response as the speed typically ranges around $10 \mu\text{m}\cdot\text{min}^{-1}$ in 3D collagen matrices (Niggemann et al. 1997)

I.2.2 - Collective cell migration

In addition to single cell migration, cells can also move collectively. This strategy is notably used in tissue remodeling, like during morphogenesis or wound healing. Collective migration can take different forms, cells can move as a mono or multilayer, in 2D or 3D, as sheets, strands or streams. They can form ducts and create an internal lumen, as for example during angiogenesis or the mammary gland formation. They can also move as small clusters; *Drosophila*'s border cells use this strategy to reach the eggs in the ovary (Niewiadomska, Godt, and Tepass 1999). Collective migration is also used by cancer cells and all these strategies have been observed during tumor cell invasion.

If collective migration regroups very different strategies, they all have common mechanisms in their organization. Maintenance of cell-cell junction allows the cells to remain cohesive and to migrate as a cohort. These junctions also allow to coordinate the actin cytoskeleton within the cohort, and it remains a supracellular structure with coordinated movement.

The type of locomotion used by cell aggregates is similar to the mesenchymal cycle movement with polarization, protrusion, adherence and retraction. Cells are collectively polarized with leader cells at the front having distinct role from followers at the rear. Leader cells are specialized in protrusion, guidance and matrix degradation. Then, follower cells provide the retraction movement through coordinated cytoskeleton contraction. It has been recently proposed that, in certain conditions, collective cell migration can rely on amoeboid-like mechanisms with global aggregates movement being based on pure actomyosin contraction rather than actin polymerization.

In addition, it has been demonstrated that collectively migrating cancer cells can switch to individual cell migration, if properly challenged (Aman and Piotrowski 2010).

I.3 - MECHANISMS OF CELL LOCOMOTION

I will here describe in depth the mechanisms of mesenchymal migration of single cells, as it is the best understood mode of migration and also the mode of migration of the breast cancer cells used during my PhD. The engine of cell locomotion is the actin cytoskeleton that produces forces required to move. However, this engine would be useless if it was not somehow

connected to the substrate on which the cell moves. This is allowed through adhesions to the extracellular matrix (ECM) that use specific receptors to bind the components of the ECM. The interplay between adhesions and the actin network generates a cycle of events that drives migration. First, the cell polarizes, under the control of extracellular cues, to define a front and a rear. Then, the cell extends a protrusion at its leading edge. This protrusion is stabilized by generation of new adhesions to the ECM. Finally, the cell's rear retracts to allow forward translocation (Figure 3). In addition to these critical steps, cells sometimes need to degrade the ECM, particularly in dense, 3D environments, in order to excavate a passageway for migration. I will here mostly describe the role of the actin network and adhesions in mesenchymal cell migration.

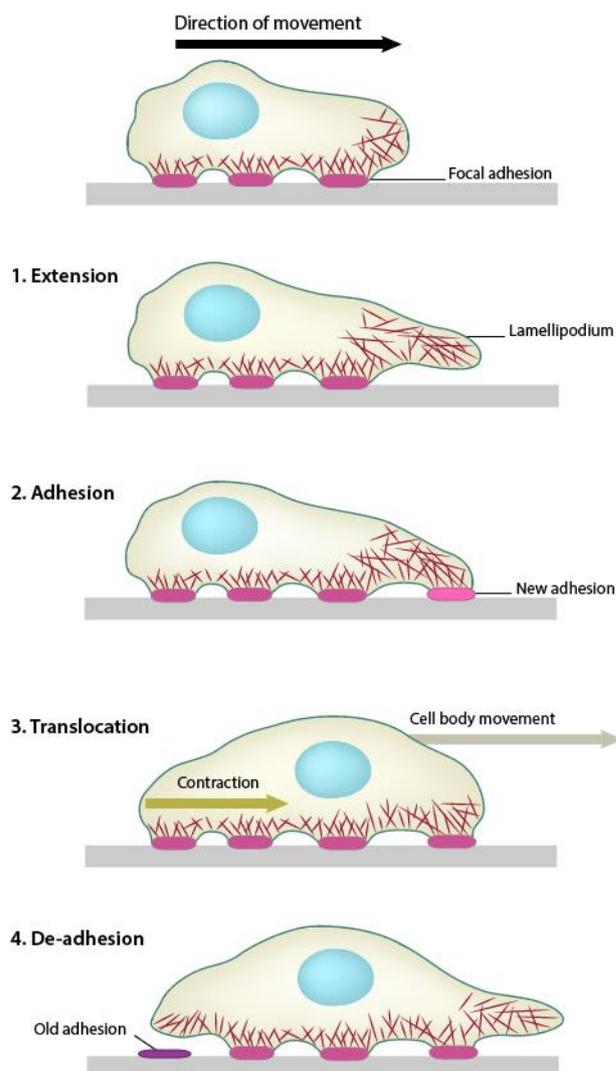


Figure 3. Mesenchymal cell migration is a repetition of 4 steps.

- 1) Actin polymerization extend the lamellipodia at the leading edge.
- 2) New adhesion forms in the new lamellipodia.
- 3) Actin contraction induce a translocation of the cell body toward the front
- 4) Retraction of the rear leads to detachment of old adhesions.

(From <https://www.mechanobio.info>)

I.3.1 - The actin cytoskeleton: the engine of cell migration

The actin cytoskeleton is the engine of cell migration by providing the forces required to move. A branched actin network polymerizes at the front of the cell to generate forces that push the plasma membrane of the leading edge forward. In addition, actin stress fibers provide contractile forces required to translocate forward the rear of the cell. Because of these numerous roles, actin regulation involves many proteins that control actin filament assembly, length, renewal or elongation speed among other characteristics (Figure 3).

I.3.1.1 - Actin and actin regulators

Actin is a globular protein that exists as a monomeric, or globular form (G-actin) that can assemble into a double helix-shaped filament (F-actin) (Holmes et al. 1990). Actin filament formation starts with a nucleation step, the assembly of three actin monomers. Actin monomers have a weak affinity for each other, and in the cell the nucleation step is both helped and regulated by actin nucleators. After initial nucleation, G-actin is added at the barbed end (+ end) of the growing filament during the elongation step. Importantly, barbed-ends are oriented towards the plasma membrane. Thus, a new monomer has to “squeeze” between the plasma membrane and the extremity of the filament in order to be incorporated. This is what actually produces the force that pushes the membrane forward. However, because the plasma membrane opposes a resistance to the pushing force, the incorporation of new monomers at the barbed-end actually results in a net displacement of the filament towards the cell center in a process known as actin retrograde flow. It is thus necessary to connect actin filaments to adhesion structures so that the pushing force can result in a net forward movement of the plasma membrane (Figure 4).

Several proteins regulate actin polymerization. Profilin is bound to cytoplasmic actin monomers. It prevents nucleation and leave the monomer upon its integration into the filament (Pollard and Cooper 1984). ADF/cofilin binds to actin monomers in the actin filament and promotes filament severing by creating a fragile zone (Andrianantoandro and Pollard 2006). Capping proteins bind to the barbed end, until actively removed, and block the filament elongation (Bearer 1991). It helps to orient global actin polymerization toward the plasma membrane. Cortactin activates the Arp2/3 complex, a central actor in the formation of the branched actin network, and stabilizes actin branched network (Lai et al. 2009).

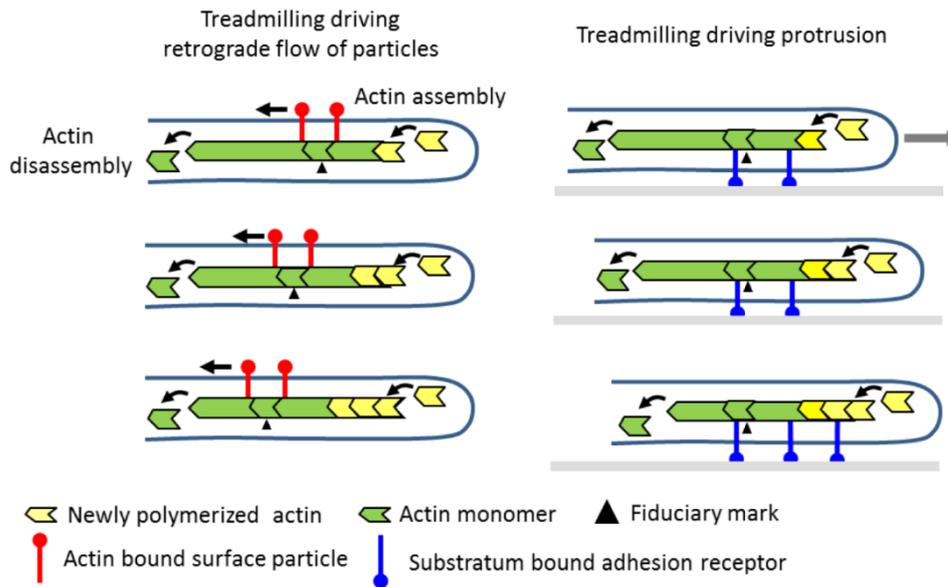


Figure 4. In the lamellipodia, actin filaments polymerize by insertion of new monomers at the extremity facing the plasma membrane. Filaments elongation thus pushes the plasma membrane forward. A) When the actin network is not engaged with the substratum, this leads to an actin retrograde flow and no forward migration. B) When the actin network is engaged with the substratum this lead to membrane displacement and initiate cell migration. (From Lee, 2018)

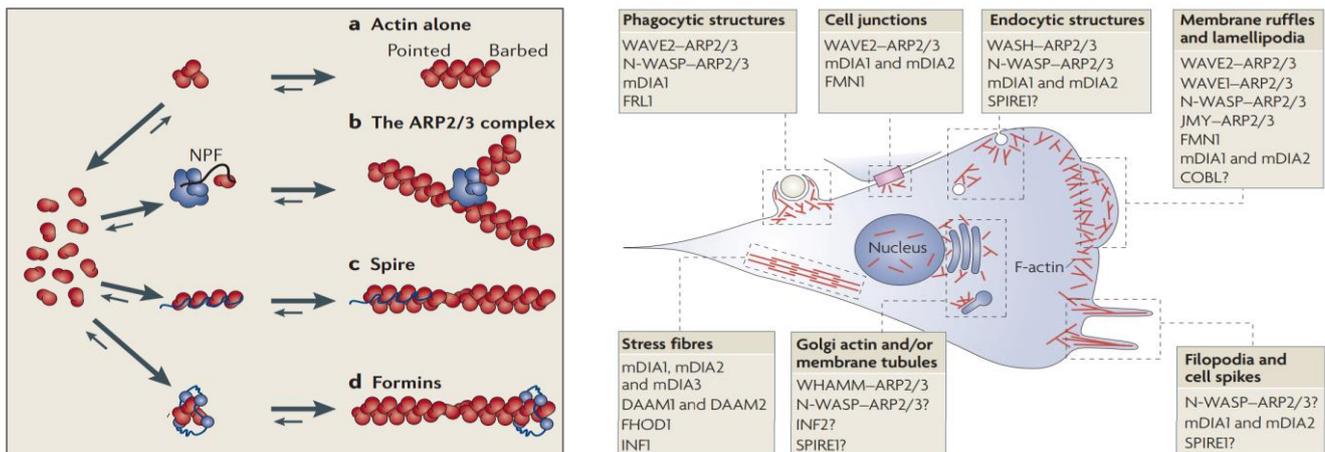


Figure 5. The different actin nucleators and their roles in the cells. A) Arp2/3 induces branched actin network assembly while formins (mDia/FMN), Spire and COBL and WH2 induce filaments formation. B) Each actin nucleator and its activator only localize in specific areas of the cell where they fulfill precise functions. (From Goley and Welch, 2006; Campellone and Welch, 2010)

Actin nucleators generate the initial the F-actin nucleus from which actin filament elongates (Figure 5). They control the future network organization and regulate actin organization by skewing the limiting steps. Arp2/3 is the only nucleator able to generate a branched actin network. It fulfills many roles and can be found in the lamellipodia but also associated at clathrin-coated structures and on endosomes. It is constitutively inactive and can be activated by nucleation promoting factor through a conformational change (Rouiller et al. 2008). Once activated, Arp2/3 polymerize a new filament on the flank of a pre-existing with at 70° orientation as compared to the mother filament (Mullins, Heuser, and Pollard 1998). This is what produces the branched network. Formins are another family of actin nucleators and are notably involved in the generation of long filaments as seen in stress fibers or in filopodia (Goode and Eck 2007). Other nucleators involve WH2 domains that bind actin monomers to promote nucleation and filament elongation. Spire (Quinlan et al. 2005) or Cordon bleu (Ahuja et al. 2007) are two members of this family.

Actin nucleators are under the control of nucleation promoting factors (NPFs) that all contain a Verprolin Homology/Cofilin/Acidic (VCA) domain. The VCA domain binds to an actin monomer and activate Arp2/3 by a conformational change (Espinoza-Sanchez et al. 2018). NPFs families have distinct roles and are spatially restricted (Figure 5). One of them, the WAVE complex, regulates the lamellipodia formation and is itself activated by the small GTPase Rac (Chen et al. 2010). It is recruited at the plasma membrane by phosphatidyl-inositol-3,4,5-phosphate (PIP3) (Oikawa et al. 2004). WASP and N-WASP participate in endocytosis and podosome formation (Benesch et al. 2005; Merrifield et al. 2004; Mizutani et al. 2002). They are activated by phosphorylation and binds to PIP2 and cdc42 (Higgs and Pollard 2000; Rohatgi et al. 1999). Finally, WASH regulates endosome fission and WHAMM is involved in trafficking events. In all cases, NPFs are recruited and activated by Rho GTPases like Rac at the level of membranes. As a consequence, actin polymerization always occurs against a membrane.

I.3.1.2 - The lamellipodia

In migrating mesenchymal-like cells, actin polymerization at the leading edge occurs in a very thin area at the very edge of the cell. This structure is called the lamellipodia and is home to a dense branched actin network (Svitkina and Borisy 1999; Figure 6). Its polymerization requires few necessary proteins but much more regulators. This actin network polymerizes

against the plasma membrane and, following the Brownian ratchet model (Peskin, Odell, and Oster 1993), thermal agitation makes enough space between actin filament and the plasma membrane for the addition of actin monomers at the barbed end of polymerizing filaments. This generates the force that pushes the membrane forward and allows the cell to protrude.

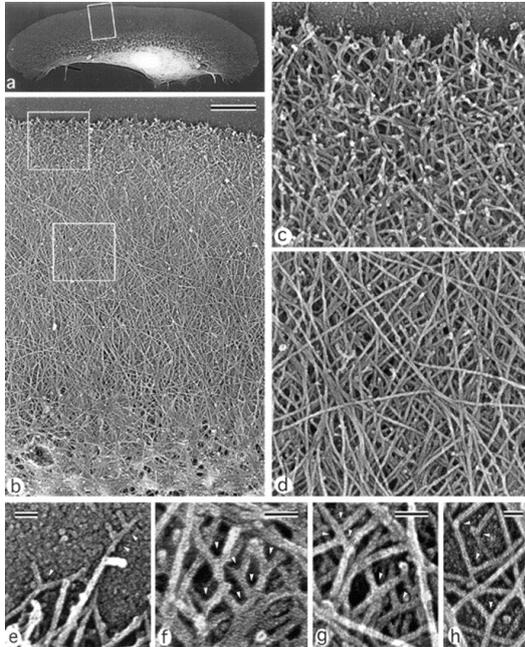


Figure 6. The lamellipodia is home to a dense branched actin network. a) EM replica of an unroofed migrating keratocyte reveals a large lamellipodia b) Overall imaging of the lamellipodia c) Branched actin network close to the membrane d) Non-branched actin filament in the center of the cell e-h) Actin branching by Arp2/3. Bars: (b) 1 μm ; (e-h) 50 nm (From Svitkina et al., 1997)

In 2000, Pollard et al proposed a model for actin organization in the lamellipodia that is still widely accepted today. In this model, an external stimulus leads to local PIP2 production and consecutive GTPases activation. This leads to NPFs activation and thus to Arp2/3 driven branched actin polymerization. Elongation factors are enriched in the front. Actin filaments elongate from their barbed end until capping proteins stop the elongation. Then, as filaments age, they become a target of cofilin that sever them. Profilin binds the monomeric actin and catalyzes the exchange of ADP to ATP thus refiling the pool of actin monomers ready for a new filament formation. Lamellipodia formation is thus an equilibrium between polymerization at the front and severing/depolymerization at its back (Figure 7).

In the absence of adhesion to the substrate, branched actin polymerization and treadmilling only lead to a net actin retrograde flow oriented towards the cell center. If adhesions physically connect the immobile substrate to the treadmilling, branched actin network, a molecular clutch transforms the retrograde flow into forces applied against the plasma membrane, eventually leading to the membrane being pushed forward and, in fine, to cell protrusion.

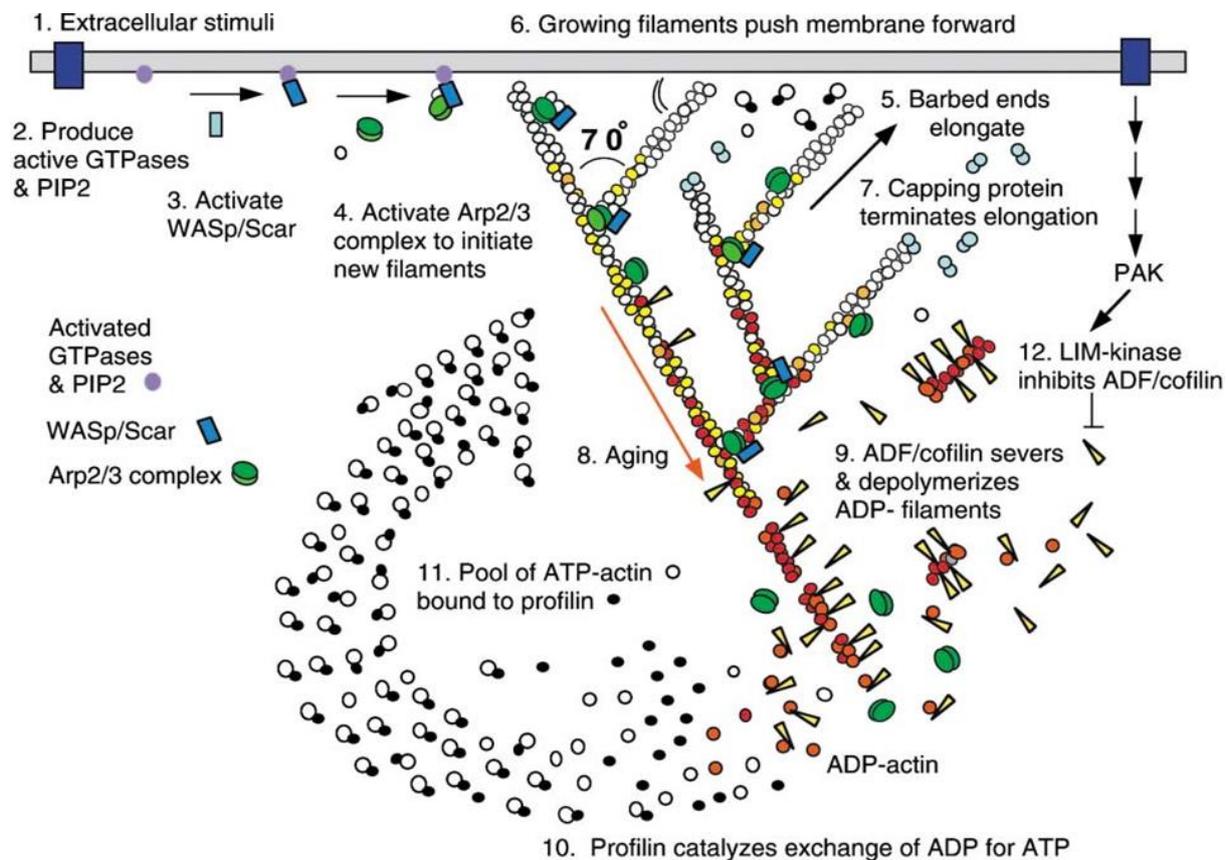


Figure 7. Proposed model for actin organization in the lamellipodia. An external stimulus leads to local PIP2 production and consecutive GTPases activation. This leads to NPFs activation and thus to Arp2/3 driven branched actin polymerization. Elongation factors are enriched at the front. Actin filaments elongate from their barbed-end until capping proteins stop the elongation. Then, as filaments age, they become a target of cofilin that severs them. Profilin binds the monomeric actin and catalyzes the exchange of ADP to ATP thus refiling the pool of actin monomers ready for a new filament formation. Lamellipodia formation is thus an equilibrium between polymerization at the front and severing/depolymerization at its back. (From Pollard and Borisy, 2003)

I.3.1.3 - Other actin structures in cell migration

Besides the lamellipodia that is the protruding region of the cell, other actin-rich structures play important functions during cell migration. Behind the lamellipodia, is a larger region called the lamella in which nascent adhesions mature to form proper focal adhesion structures. It is also the region where actin stress fibers are formed and play a role in migration. A very important player of cell migration is found in this area, and is actually excluded from the lamellipodia: myosin-II. Myosin-II is a molecular motor that, opposite to other myosins, do not move along actin filament but is able to slide two anti-parallel filaments through

hydrolyzing ATP and changing conformation (Rayment et al. 1993; Figure 8). The HC links F-actin and hydrolyze the ATP. Myosin-II tail can bind to other myosin-II tails to form fibrils. The RLC regulate myosin activity through its phosphorylation state. Myosin-II fibrils, together with anti-parallel actin filament, form contractile complexes (actomyosin) and their activity is required for cytokinesis, muscle contraction or stress fibers contraction. I will here describe different actin-rich structures playing a role in cell migration, most of which also heavily depend on the action of myosin-II. It is important to keep in mind that most of these structures (including the lamellipodia) have mostly been described in cells migrating on 2D substrates and, consequently, their description is relevant only in this context.

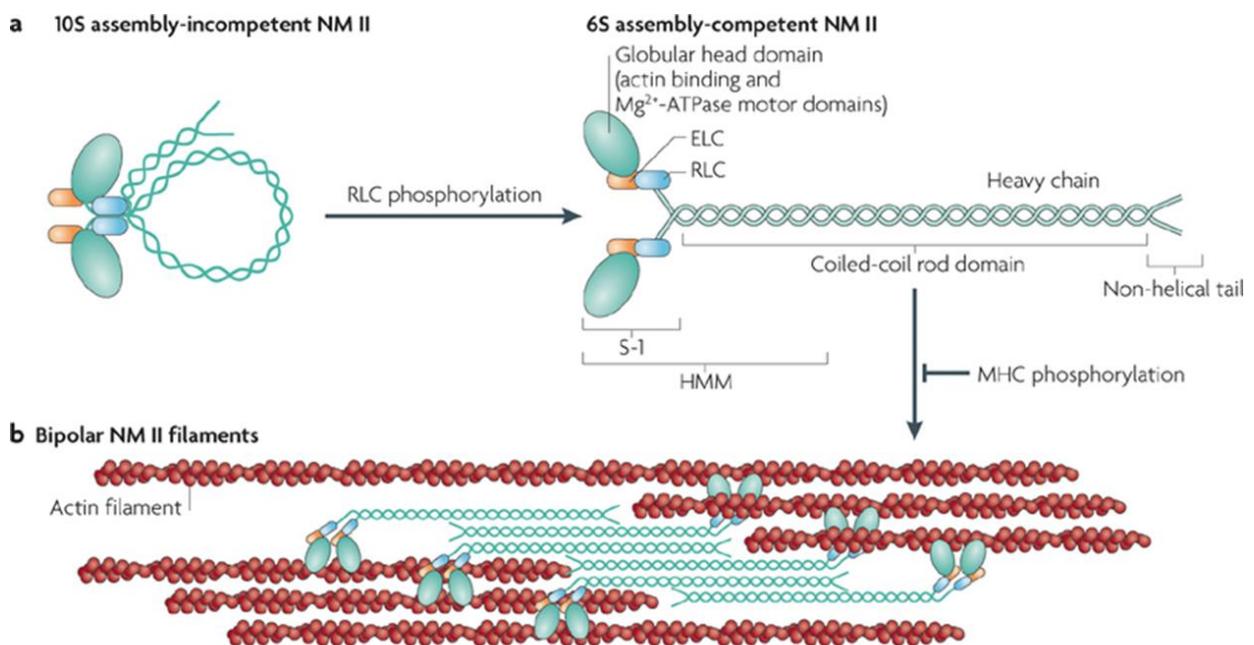


Figure 8. A) Myosin-II is composed of two head chains (HC), two regulatory light chains (RLC) and two essential light chains (ELC). Upon RLC phosphorylation myosin II adopts its unfolded active conformation. B) Myosin dimerizes and binds actin filaments; the action of myosin dimers leads to actin fiber contraction. (From Vicente-Manzanares, 2009)

The actin cortex

The actin cortex is a 100 nm thin layer of reticulated actin that is just below the plasma membrane (Bray and White 1988). It participates in establishing/maintaining the cell shape and resistance to applied forces. Myosin-II-mediated contraction is responsible for the cortical tension. Its main function is during mitosis as it helps the cell to round up, but it also plays a role in migration. The actin cortex contains many actin bundlers and crosslinker like fascin or

α -actinin. It contains both actin bundle and branched actin and it is mainly nucleated via Arp2/3 and formins (Bovellan et al. 2014). It is bound to the plasma membrane by ezrin, radixin and moesin (ERM proteins). It is a very dynamic structure that contain many actin regulators. Cortical actin is particularly important during amoeboid migration as the dynamic interplay between the cortex and the plasma membrane is responsible for bleb formation and resolution (Blaser et al. 2006).

Actin stress fibers

Stress fibers are long cables (up to 10 μm) of antiparallel actin filaments that are linked together by myosin-II and α -actinin. In addition to myosin-II and α -actinin, many actin binding proteins are associated with stress fibers, including the actin recruiter tropomyosins. These proteins have a fast turnover, making stress fibers dynamic structures (Tojkander et al. 2011). Stress fibers are found from the lamella to the rear of the cell (Cramer, Siebert, and Mitchison 1997). Thanks to myosin sliding along antiparallel actin filaments, stress fibers are contractile structures and are, most of the time, under tension. They can be associated to focal adhesions (FAs) at one or both of their extremities and the tension generated by stress fibers helps FAs to mature (Vogel 2006). They participate in mechanotransduction, transmit forces and are necessary for the retraction of the rear of the cell during migration.

Stress fibers can be divided into three subtypes that also represents different maturation steps of these structures (Figure 9). Transverse arcs are located in the lamella and oriented parallel to the leading edge. They are not associated with adhesion sites but their contraction participates to the actin retrograde flow that is also observed in the lamella (Zhang et al. 2003). Dorsal stress fibers are located on the dorsal side of the cell, perpendicular to the direction of leading edge extension and are only attached to the substrate through one adhesion site. Ventral stress fibers are anchored to FAs at both their can thus exert strong contractile forces. Ventral stress fibers are at the back and promote rear contraction, detachment and displacement (Mitchison and Cramer 1996).

Stress fibers arise from existing actin filaments coming from the lamellipodia reorganization. Cofilin-severed actin filaments at the rear of the lamellipodia start to be enriched in myosin-II and α -actinin when entering the lamella, and to reorganize into transverse arc of antiparallel actin filament bundles (Burnette et al. 2011; Shemesh et al. 2009). Nucleation

activity associated with transverse arc dynamics relies on formins (mDia and FHOD1) via the Rho/ROCK pathway (Watanabe et al. 1997). Dorsal fibers come from actin polymerization at adhesion sites. Ultimately, myosin-II mediated contractions leads to transverse arc and dorsal fibers reorganization into ventral stress fibers (Hotulainen and Lappalainen 2006).

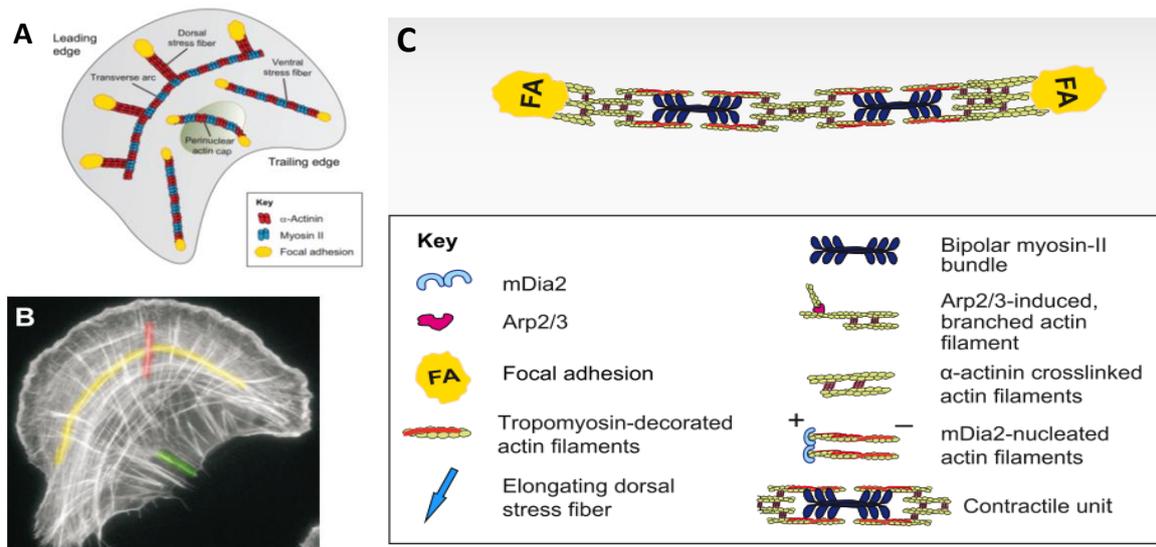


Figure 9. A) In the cell, stress fibers can be sorted into 3 categories: dorsal stress fibers that have one extremity associated with FAs, ventral stress fibers that have two extremities associated with FAs and transverse arc that are not associated to FAs. B) Actin staining showing dorsal stress fibers (red), ventral stress fibers (green) and transverse arc (yellow). C) Structure and composition of a ventral stress fiber. (Adapted from Tojkander et al., 2012)

Filopodia

Filopodia are small, pointy membrane outgrowth that form mostly in the lamellipodia. They serve to sense and explore the cell environment. They form in contact zones with the ECM and their bundled, parallel actin filaments structure is connected to nascent adhesions (Steketee and Tosney 2002). The particular organization of actin filaments in filopodia is controlled by fascin that can only bundle parallel filaments. Formins, located at the tip of filopodia, ensure further filament elongation (Yang and Svitkina 2011). Myosin X transports receptors to filopodia tips for effective sensing (Jacquemet, Hamidi, and Ivaska 2015). Among these receptors are integrins, cell-cell receptors, growth factor receptors and chemokines receptors. Filopodia are mostly used to sense the environment, to transduce external stimuli into signaling. They are extensively used at the beginning of cell spreading (Albuschies and Vogel 2013) but

also during cell migration where they have been proposed to regulate contact inhibition (Arjonen et al. 2014) or to recognize the ECM topography. They have also been proposed to produce retraction forces (Jacquemet, Hamidi, and Ivaska 2015).

I.3.2 - Adhesion structures

Adhesions to the ECM are critical for cell migration as they allow the actin machinery to be physically connected to the ECM. This allows the actin retrograde flow to be slowed down and consequently, the barbed ends of lamellipodia actin filaments to efficiently push the plasma membrane forward. Adhesions are formed around ECM receptors but also contains many cytosolic proteins that are critical for adhesion sites functions. These proteins fulfil different functions and are recruited at adhesion sites through direct or indirect binding of the cytosolic tails of ECM receptors. To date, different types of adhesion complexes have been described, that, for most of them, display a similar composition and depends on connections with the actin cytoskeleton. Besides the classical focal adhesions (FAs), other structures sharing to some extent similarities with FAs such as invadopodia, podosomes and hemi-desmosomes can exist in some cells and, in addition to adhesion, fulfil some specialized functions. More recently, new types of adhesion structures have been proposed that do not rely on actin but that contain clathrin and clathrin-associated endocytic machinery components. I will here describe the most canonical adhesion structures (that will be termed “focal adhesions” for practical reasons) before briefly evoking clathrin-coated structures.

I.3.2.1 - ECM receptors

Several families of ECM receptors co-exist in cells, but the integrin family is widely considered to be the main one as integrins bind to most of the ECM components. In addition to integrins, other receptors such as the discoidin domain receptors, GPV1, LAIR-1 and the mannose receptor can take part in adhesion (Leitinger and Hohenester, 2007). On the top of their role ECM binding, these receptors also activate downstream signaling pathways with important physiological consequences.

Discoidin domain receptors

Discoidin domain receptors (DDR) are a family of collagen receptors containing two members, DDR1 and DDR2 (Shrivastava et al. 1997). DDR1 is mainly found in epithelial cell and DDR2 in mesenchymal cells. They can recognize different collagen types, but both bind to collagen type I. They bind to the GVMGFO collagen region (Xu et al. 2000) but only in the triple helical collagen configuration (Vogel et al. 1997).

DDRs are the only Receptor Tyrosine Kinases (RTK) that bind to the ECM. Like other RTKs DDrs dimerize upon ligand binding. This leads to autophosphorylation of the receptors and activation of downstream effectors like Cdc42, ERK1/2-MAPK or members of the STAT family (Ongusaha et al. 2003; Wang et al. 2006). Interestingly, DDrs require a long ligand presentation, 2 hours, and remain activated for 16 hours (Shrivastava et al. 1997; Vogel et al. 1997). They are involved in adhesion, migration, ECM remodeling and proliferation.

Integrins

The integrin family of ECM receptors is mainly involved in cell-matrix adhesion but can also play a role in cell-cell adhesion. They serve to analyze the properties and composition of the cell environment and they transform these physical and chemical external stimuli into the adapted intracellular response.

Integrins role can be divided into two part as they are both adhesion molecules and signaling receptors. As adhesion molecule they link the cell to the ECM and initiate the formation of large adhesion complexes. As signaling receptors they control cell survival, cytoskeleton dynamic, cell cycle progression, polarization or mechanosensing. Integrins subunit expression level can vary over time or depending of the cell type and this defines to what component of its surrounding matrix the cell can bind.

Integrins are heterodimer composed of non-covalently bound α and β subunit. α subunits are composed of around 1000 amino acids and β subunits a bit shorter, being composed of around 750 amino acids. Their heterodimerized, large N-term extracellular domains form the structural entity endowed with the capacity to bind to ECM proteins and thus provide dimer-ligand specificity. The transmembrane domains regulate heterodimerization. Integrin dimerization initially occurs in the endoplasmic reticulum (ER), prior to reaching the cell

surface (M. J. Humphries 2000). The short C-term intracellular domains provide binding to actin and other adhesion proteins. Integrins are non-enzymatic and thus rely on their partners for signaling.

18 α and 8 β different subunits can associate into a total of 24 different heterodimers reported so far (Sheppard 2000). A specific heterodimer is mostly associated to one type of ECM component. On the opposite, a given ECM protein can be recognized by several heterodimers (Humphries, Byron, and Humphries 2006; Figure 10A). As a consequence, a cell adhering to a particular ECM protein can recruit different heterodimers to do so. As an example, collagen type I is recognized by 5 integrin heterodimers: $\alpha1\beta1$, $\alpha2\beta1$, $\alpha10\beta1$, $\alpha11\beta1$ and $\alpha X\beta2$ (Humphries, Byron, and Humphries 2006).

Integrins have been historically identified as the receptor for the RGD (arg-gly-asp) domain of fibronectin. RGD domains are also responsible for the integrins binding to vitronectin or osteopontin. Nevertheless, even if RGD sequence is found in collagen, integrins that bind to collagen actually recognize the triple helical GFOGER sequence (Knight et al. 1998), the later identified GLOGER sequence or the GROGER motif (Raynal et al. 2006). Finally, integrin's binding to its ligand is also dependent on cation such as Ca^{2+} , Mn^{2+} , Mg^{2+} that are required to support the proper structural organization of the high affinity dimers (Hu, Barbas, and Smith 1996).

Integrins can be found in two conformations (Figure 10B). In the inactive, folded conformation, the extracellular domains are bent and show a low affinity for the ligand (Nishida et al. 2006). In the active conformation, the heterodimer is unfolded and displays a very strong affinity for its ligand (Jahed et al. 2014). The dimerization of the transmembrane domains favors the inactive state (Wegener et al. 2007). Integrin activation can be regulated by different phenomenon. Inactivated integrins diffuse freely at the plasma membrane and can be activated by both ligand binding or intracellular partners like talin.

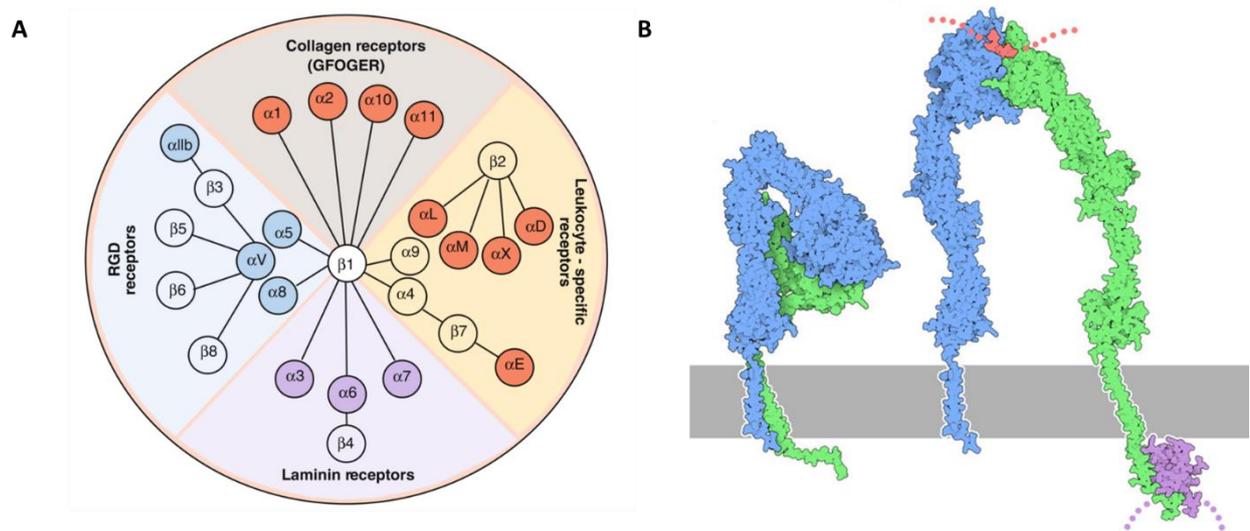


Figure 10. A) Representation of integrin-ligand interactions. Integrin-ligand interaction specificity is given by the dimer composition. (From Barczyk, 2010) B) Inactive conformation of integrin (left) and a hypothetical model of the open, active form (right), with a fibrinogen peptide in red and a talin domain in magenta. The α integrin is in blue and the β integrin is in green. (From <https://pdb101.rcsb.org/motm/134>)

I.3.2.2 - Focal adhesions

Focal adhesions (FAs) are clusters of proteins forming around activated integrins. They not only physically link the cell to the ECM but also play a major role in signaling in response to physical cues from the environment and in interacting with the actin cytoskeleton, thus playing a major role in cell migration.

General description of FAs

Many proteins take part in FAs assembly and dynamics and, together, they form what is generally described as “the adhesome”. Studies found 148 resident proteins at FAs and another 84 associated proteins (Winograd-Katz et al. 2014). FAs proteins can be divided into 4 main groups:

- Adaptor proteins, scaffold, contact protein (*eg.* talin, ILK, paxillin, vinculin)
- Cytoskeleton binding proteins (*eg.* α -actinin)
- Enzymes (*eg.* FAK, Src, PKC, PI3K)
- Small GTPases and GTPases regulators (*eg.* Rho, Rac, cdc42)

The many binding sites that are found in these proteins make FAs a very complex and interconnected network. Nevertheless improvement in imaging technics allowed to start uncovering the substructural organization of FAs (Figure 11; Kanchanawong et al. 2010). These studies have proposed an organization in three functional layers:

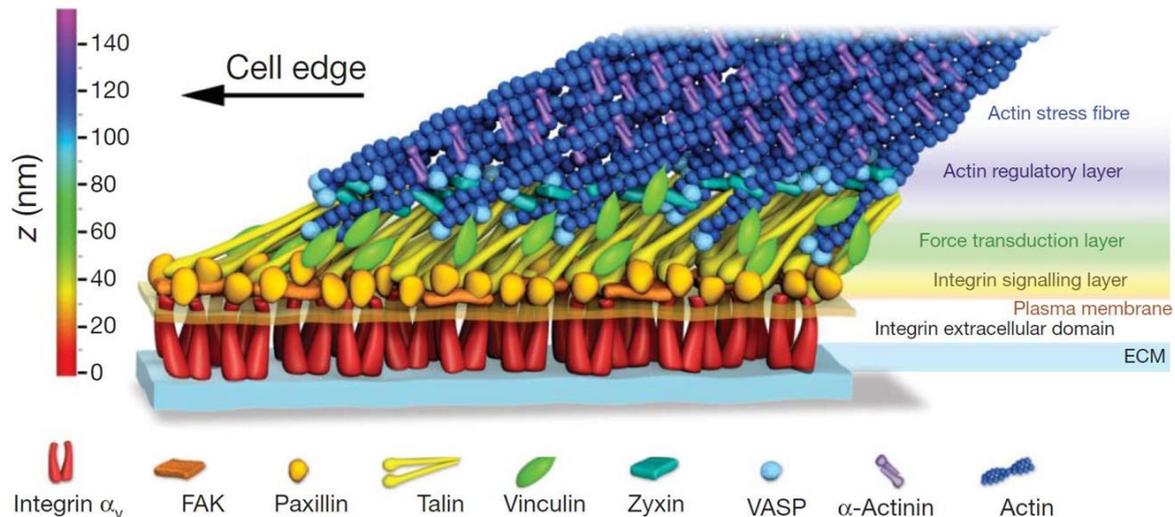


Figure 11. Focal adhesions are composed of different layers that are defined by their role, composition and distance to the plasma membrane. (From Kanchanawong et al., 2010)

- The integrin-signaling layer

This is the most plasma membrane proximal layer and comprises the cytosolic tail of integrins as well as cytosolic factors directly binding this cytosolic tail. Talin is a central regulator of integrin affinity and FAs dynamics. Talin is a large protein that adopt an elongated shape. While the head of talin is very close to the plasma membrane, the protein also spans across the two other layers of FAs. Talin binding to β integrin subunits leads to the separation of α and β cytoplasmic tails. This favors the active, high affinity state of integrin dimers and leads to strong ECM engagement (Wegener et al. 2007). Talin interacts with many other proteins including signaling proteins. It makes the first direct link with cytoskeleton as it also binds F-actin in more distal layers (Lee et al. 2004). Its four integrin binding sites participate in integrin clustering (Klapholz et al. 2015). Another central player of this layer is the Focal adhesion kinase (FAK). FAK is both a scaffold for other FAs-associated proteins and the major FAs signaling protein. Its phosphorylation by Src leads to the activation of Rho-GTPases like RhoA, cdc42 or Rac-1 (Schaller 2010). It is not required for adhesion formation or actin linking but regulates FAs dynamics (Mitra, Hanson, and Schlaepfer 2005). Paxillin is a signaling and

scaffolding protein that is recruited very early during FAs assembly. It binds and recruits kinases (eg. FAK, Src), actin binding protein (eg. vinculin), as well as regulators and effectors of the Rho family of small GTPases. Src family of kinase (SFC) is a family of tyrosine kinase proteins that are quickly activated after integrin activation (Baruzzi, Cavegion, and Berton 2008). They contribute to reinforce and mature the adhesion complex by activation of downstream kinases and adaptors (Giannone and Sheetz 2006).

- The force transduction layer

This layer comprises protein or protein domains that are sensitive to physical forces exerted at FAs. As discussed above, talin also spans this layer and is endowed with the possibility to unfold some domains when exposed to forces. This exposes new binding sites for the recruitment of other regulators and adaptors of FAs and thus directly participates in force-regulated FAs maturation. Vinculin is another important actor of this layer. It is a scaffolding protein that interacts with proteins like F-actin, talin, α -actinin, paxillin, WASP and Arp2/3. The force-induced unfolding of talin exposes vinculin-binding sites and vinculin subsequently plays a critical role in FAs maturation through recruiting additional factors and reinforcing the link between integrin and the actin cytoskeleton (Humphries et al. 2007; Saunders et al. 2006).

- The actin regulatory layer

Finally, the most membrane distal layer is the actin regulatory layer. This is where is found α -actinin, an actin filament crosslinker that participates in the formation of the actin bundle that emanates from FAs (Brown et al. 2006; Choi et al. 2008). The more α -actinin there is, the stronger is the linkage between FAs and the actin cytoskeleton (Laukaitis et al. 2001; von Wichert et al. 2003). The actin regulatory layer also contains myosin II that control both FA maturation and disassembly via actomyosin contraction (Vicente-Manzanares et al. 2007).

Maturation and life-cycle of FAs

Upon integrin clustering, FAs mature from small proteins cluster often nascent adhesions into larger structures by recruiting more and more proteins (Figure 12A). Their shape, signaling and role in adhesion evolve with their composition (Vicente-Manzanares and Horwitz 2011). First formed are nascent adhesions that can first mature into focal complexes before further maturation into proper FAs that ultimately disassemble at the rear of the cell, allowing the cell body to translocate forward (Figure 12B). These distinctions are mostly practical as FAs maturation is more a continuum than a distinct succession of discrete class of adhesions.

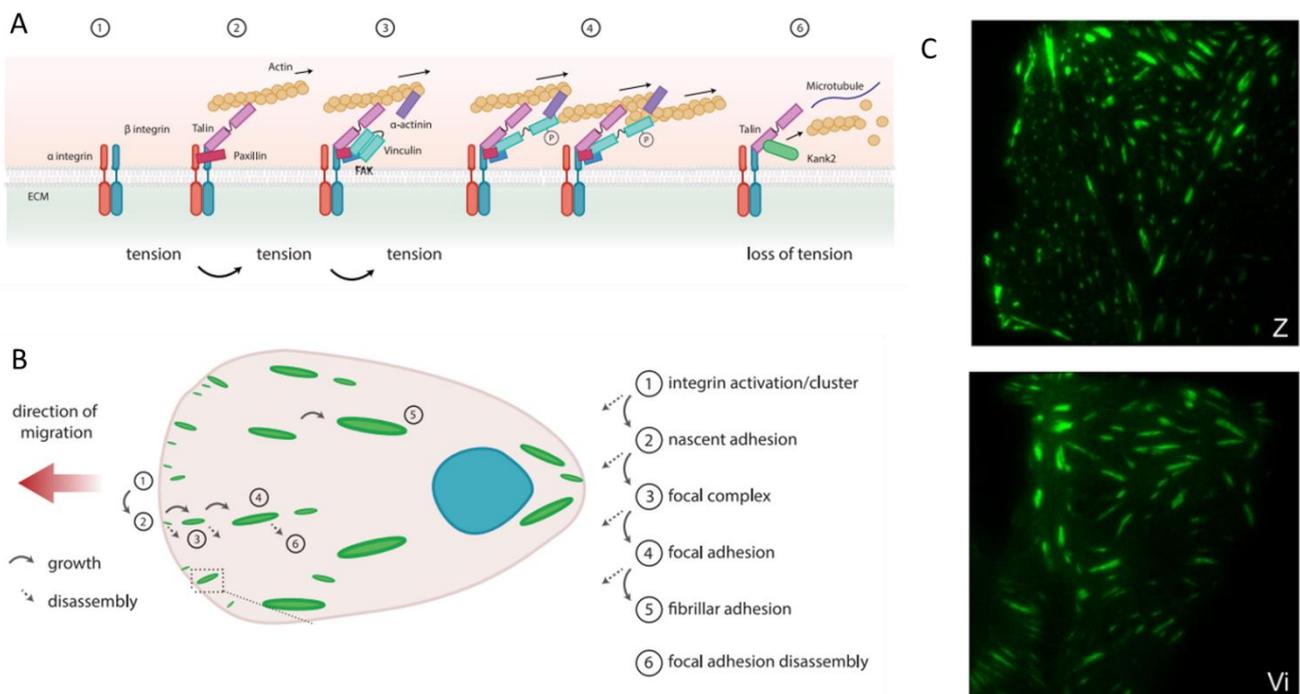


Figure 12. A) Focal adhesions mature by sequential recruitment of proteins B) As they mature and recruit proteins focal adhesions elongate. They disassemble when once at the rear of the cell. (From De Pascalis and Etienne-Manneville., 2017) C) Zyxin (Z) and vinculin (Vi) staining showing the different shapes of focal adhesion in U20S cells (From Legerstee et al., 2019)

Nascent adhesions form at clustered integrin sites as dot-shaped structures of about 200nm in diameter. Their nucleation mechanism remains unclear, but they only form at the cell front (Galbraith, Yamada, and Galbraith 2007). Nascent adhesions formation requires F-actin polymerization that occurs at the very leading edge of the cell in a structure called the lamellipodia (Alexandrova et al. 2008; Choi et al. 2008). They exhibit strong phosphorylation

capacities (at FAK, Src, Paxilin) that participate to protein recruitment and promote branched actin polymerization in the lamellipodia. They could be responsible for the strong traction force observed at the very front of the cell (Beningo et al. 2001; Munevar, Wang, and Dembo 2001).

Most of these short-lived structures (30s) (Choi et al. 2008) disappears as they are dragged backward by the actin retrograde flow that results from the intense actin polymerization against the plasma membrane of the leading edge, in the lamellipodia. The few ones associated with actin bundles can persists to form focal complexes (Oser and Condeelis 2009).

Focal complexes are up to 1 μm elongated structures. Their maturation relies on myosin II contraction (del Rio et al. 2009) and coincide with the transition from the lamellipodia to the more distal lamella. Their composition doesn't change as compared to nascent adhesions (Choi et al. 2008). Focal complexes then disassemble or mature into proper FAs.

FAs are ovoid structures of 2 μm to 10 μm long. Their turnover is quite slow with a lifetime of several minutes. The more rigid is the substrate the larger FAs are (Balaban et al. 2001). Phosphotyrosine levels are reduced over FAs maturation as the adhesion progressively loses its signaling role for a more adhesive one (Ballestrem et al. 2006). Their composition changes as compared to focal complexes with more vinculin, paxillin or α -actinin being recruited and they also start to associate with actin stress fibers. Activation of myosin II in stress fibers leads to fiber contraction and thus to more forces being exerted at FAs (Kato et al. 2001). These forces participate to the growth of FAs to a certain threshold.

Finally, integrin disengagement and adhesion disassembly at the cell's rear are required to end signaling and for the cell to move forward. FAs disassembly is driven by many mechanisms. One possible mechanism involves the protease calpain that cleaves integrins or talin (Franco et al. 2004). Myosin II-driven actin contraction can drive FAs maturation to a certain threshold as mentioned above. However, when forces become too important, they can lead to FAs disassembly by weakening integrin interaction with ECM or physically disconnecting FAs from the actin cytoskeleton (Chrzanowska-Wodnicka and Burridge 1996). In that latter case, FAs can detach from the cell and remain attached to the ECM. Microtubules also play a role in FAs disassembly, potentially through bringing a relaxing factor to FAs (Kaverina, Krylyshkina, and Small 1999). Finally, integrin endocytosis participates in FAs disassembly in a FAK- and dynamin-dependent manner (Ezratty et al. 2009; Ezratty, Partridge, and Gundersen 2005). All these mechanisms collaborate to regulate efficient FAs disassembly.

I.3.2.3 - Adhesive clathrin-coated structures

Clathrin-coated structures (CCSs) are primarily endocytic structures and will be described in greater detail in the second chapter of this manuscript. Nevertheless, because CCSs were observed in thigh contact with the substrate (Batchelder and Yarar 2010; Maupin and Pollard 1983) and to contain ECM-engaged integrins (Batchelder and Yarar 2010; De Deyne et al. 1998; Tawil, Wilson, and Carbonetto 1993), it was proposed that they could also serve as adhesion structures (Lampe, Vassilopoulos, and Merrifield 2016). This hypothesis has become more popular recently as a new type of $\alpha\text{v}\beta 5$ -enriched adhesions have been described and shown to actually correspond to a formerly identified subset of CCSs called clathrin-coated plaques. The formation and dynamics of these atypical adhesions are independent of actin but depends on clathrin and clathrin-adaptors that recruits integrins such as Numb, Dab2 and ARH. Although seemingly not connected to the actin cytoskeleton, these structures are mechanosensitive as they assemble as a function of the substrate rigidity (Baschieri et al. 2018).

In addition, our team also recently reported the existence of another type of clathrin-based adhesions (Elkhatib et al. 2017). These structures are called Tubular Clathrin/AP-2 Lattices (TCALs) because they form at contact sites with collagen fibers and wrap around and pinch the fibers. TCALs are enriched in $\beta 1$ integrins and are used by cells migrating in 3D to grab collagen fibers thus facilitating the stabilization of long cell protrusions, in coordination with FAs.

These atypical adhesive structures will be discussed in greater detail in the second part of this manuscript.

I.3.3 - Orientation of cell migration

Cells that migrate not only move, but, very often, they actually move in a direction that is controlled by both external and internal factors. Cells can sense and interpret many kinds of external physical and chemical cues. For instance, cells can migrate by following rigidity gradients (eg. durotaxis; Lo et al. 2000), electric fields (eg. galvanotaxis; Zhao et al. 1996) gradients of soluble attractants (eg. chemotaxis; McCutcheon, 1946) or substrate-bound factors (eg. haptotaxis; Carter 1965). Directed cell migration is a key process in embryogenesis where it allows cells to move to their final destination (Reig, Pulgar, and Concha 2014). It is also central to immunity (Luster 1998) or nervous system wiring (Hatten 2002). I will here describe

more specifically the cases of chemotaxis and haptotaxis because they are the most prominent forms of directed cell migration in the organism and because it is at the heart of my PhD project.

I.3.3.1 - General mechanisms of chemotaxis and haptotaxis

Many different kinds of secreted proteins have been shown to be able to attract cells: growth factors (EGF, FGF, VEGF, IGF-1, PDGF, HGF, TGF- β ...), chemokines (CCR1, CCR2, CCR3, CXR1, CXR3...) and matrix proteins (fibronectin, vitronectin, collagen I...). However, an isotropic distribution of these cues leads to random migration. In order to orientate cell migration, extracellular cues need to be asymmetrically distributed, in a gradient. The gradient depth also needs to be adequate, as the difference of cues concentration at the front versus the rear of the cell must be sensed with sufficient sensitivity by the cell. Indeed, directed cell migration implies that the asymmetrically distributed external cues are sensed and integrated into signaling pathways that polarizes the migration machinery inside the cell (Parent and Devreotes 1999; Vorotnikov 2011).

Several chemotaxis models have been proposed and are not necessarily mutually exclusive depending on chemoattractants and cell types considered. The “chemotaxis bias” model is based on a proposed stabilization of the protrusion facing the stronger concentration of chemoattractant (Andrew and Insall 2007; Arriemerlou and Meyer 2005; Insall 2010). In this model the cell randomly generates lamellipodia in every direction. If the lamellipodia contact enough chemoattractant it will stabilize, and otherwise it will retract. In the “compass” model, the lamellipodia only form towards the more concentrated area of the gradient as a consequence of local accumulation of second messengers following receptor signaling (eg. PIP3) (Rickert et al. 2000; Swaney, Huang, and Devreotes 2010; Figure 13).

I.3.3.2 - Regulation of directed migration

Actin is the engine of locomotion and this engine can be steered by external factors in order to produce a directed migration phenotype. These cues are sensed by cell-surface receptors that transduce intracellular signals that, collectively, continuously reshape the organization of the actin network in order to move in a given direction. Classically, chemoattractant bind to two main receptor families: 1) G protein-coupled receptors (GPCRs)

and, 2) receptor tyrosine kinases (RTKs). Once activated by their ligands, these receptors triggers intracellular signaling pathways by recruiting different adaptors and effectors. Chemoattractants usually trigger two signals: 1) a locally restricted activating signal and, 2) a largely diffusing inhibiting signal (Xiong et al. 2010) thus ensuring a local control on the actin machinery.

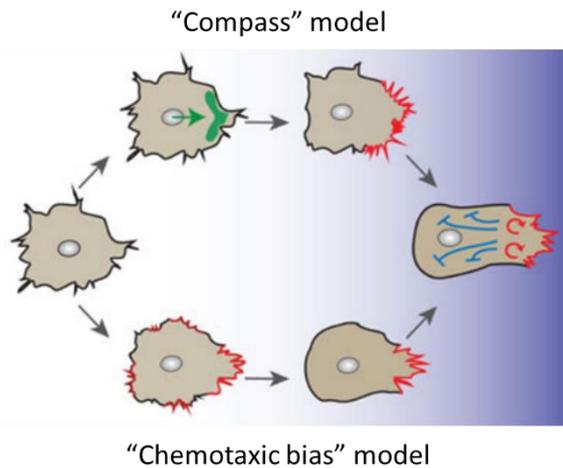


Figure 13. Two model coexist to explain chemotaxis. A) In the “chemotaxis bias” model the cell randomly generates lamellipodia in every direction. If the lamellipodia contact enough chemoattractant it will be stabilized, and otherwise it will retract. B) In the “compass” model, the lamellipodia only form towards the more concentrated area of the gradient as a consequence of local accumulation of second messengers following receptor signaling. (From Reig et al., 2014)

One of the most important signaling factor generated upon receptors activation is phosphatidylinositol(3,4,5)-trisphosphate (PIP3) (Sasaki et al. 2000). The phosphoinositide 3-kinase (PI3K) is produce as a consequence of both GPCRs and RTKs activation that control a localized PIP3 production and accumulation at the cell front (Figure 14). Many actin regulators have Pleckstrin Homology (PH) domains that can interact with PIP3 (Yin and Janmey 2003). While PI3K produces some PIP3 at the cell front, the phosphatase PTEN that accumulates at the cell rear hydrolyzes PIP3 into PIP2 thus reinforcing cell polarization (Funamoto et al. 2002; Iijima and Devreotes 2002). Receptor activation also leads to calcium and proton entry into the cell. Calcium is an important second messengers that also accumulate at the front during migration (Brundage et al. 1991). In addition, pH gradient within the cell is also known to modulate actin related proteins such as talin, cofilin or cdc42 (Frantz et al. 2007) and seems to be implicated in many directed migration (Martin et al. 2011; Tarbashevich et al. 2015).

Ultimately, these signals regulate and asymmetrical activation of RhoGTPases. For instance, Rac and cdc42 activation at the front leads to lamellipodia and filopodia formation. At the back of the cell, Rho regulates stress fiber contraction and rear cell retraction. The mutual Rac1 and RhoA antagonism reinforce the polarization (Bustos et al. 2008; Ohta, Hartwig, and Stossel 2006). (Figure 15)

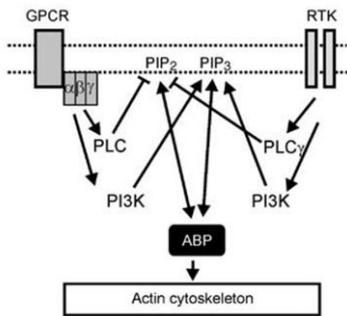


Figure 14. Upon chemoattractant binding, both GPCRs and RTKs activate PLC and PI3K leading to local enrichment of PIP₃ at the plasma membrane. This local enrichment in PIP₃ induces a local recruitment of actin nucleator ultimately leading to localized actin polymerization and directed migration toward the chemoattractant. (From Kedrin et al., 2007)

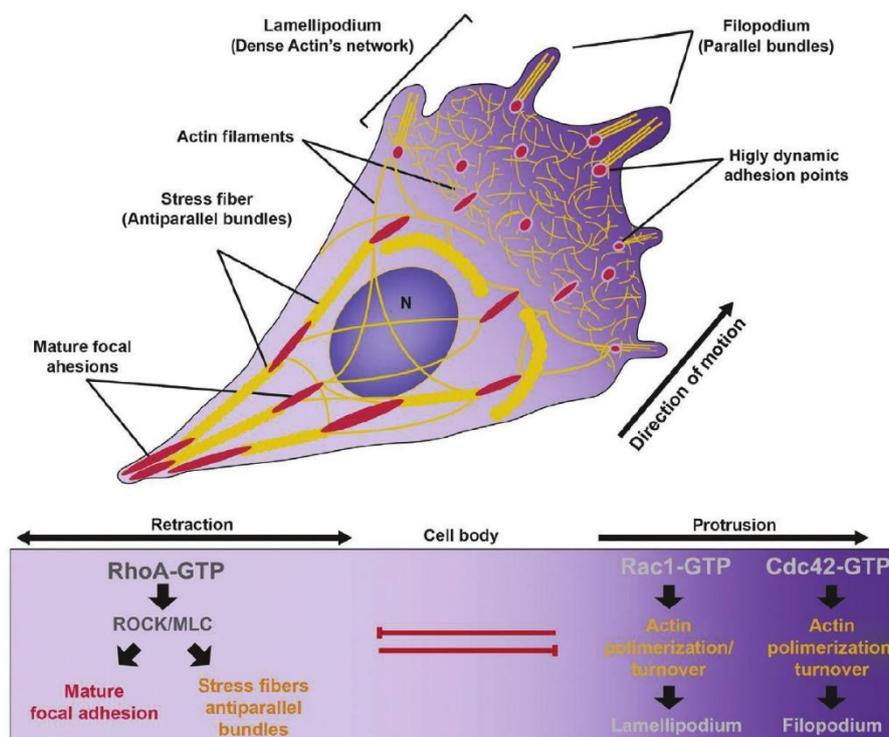


Figure 15. Chemoattractant gradients lead to asymmetrical activation of RhoGTPases. Rac and cdc42 activation at the front leads to lamellipodia and filopodia formation. At the back of the cell, Rho regulates stress fiber contraction and rear cell retraction. The mutual Rac1 and RhoA antagonism reinforce the polarization. (From Barriga and Mayor, 2015)

Microtubules also participate in directed cell migration. Microtubules regulate proteins and mRNAs traffic, RhoGTPases and FAs turnover (Tran et al. 2007). The microtubule organization center (MTOC) is oriented toward the front of migrating cell to send microtubules to the leading edge (R. Li and Gundersen 2008). If microtubules are not required to establish cell polarity they participate in its maintenance (Etienne-Manneville 2013).

Haptotaxis towards substrate-anchored growth factors or chemokines is believed to rely on similar mechanisms. However, in the case of haptotaxis towards ECM components, integrins, rather than GPCRs and RTKs are used to sense the gradient (Oudin et al. 2016). In the specific case of a fibronectin gradient, fibronectin binding to $\alpha5\beta1$ leads to Mena recruitment on their cytoplasmic tails. As Mena is a member of the Ena/VASP family that promotes actin filaments elongation, this leads to localized actin reorganization. Besides a few specific examples, and as mentioned above, haptotaxis is poorly studied and understood but is generally believed to rely on similar mechanisms as chemotaxis.

Besides a few specific examples, and as mentioned above, haptotaxis is poorly studied and understood but is generally believed to rely on similar mechanisms as chemotaxis. Nevertheless, a recent study (C. Wu et al. 2012) compared the migration mechanisms involved in chemotaxis and haptotaxis in 2D. They used Arp2/3-depleted fibroblasts to investigate the role of lamellipodia in migration in response to gradient of soluble PDGF or substrate-bound matrix proteins. They found that cells lacking Arp2/3 and lamellipodia could still migrate toward a soluble PDGF gradient, most likely using filopodia sensing. On the opposite, Arp2/3 and lamellipodia were shown to be required to follow a substrate-bound gradient of matrix protein. Nevertheless, this study doesn't allow to draw a general mechanism of chemotaxis versus haptotaxis as different chemotactic cues were used (growth factor vs matrix proteins) and these two families of migrating cues bind to very different receptors. It is likely that, in addition to the way the ligands is presented to the cell (soluble or attached to the substrate), the mechanism driving directed migration is specific to the class of chemoattractant used in the different assays.

I.4 - THE EXTRACELLULAR MATRIX

Cell migration occurs on a substrate that, *in vivo*, is the ECM. Even *in vitro*, cells do not migrate directly on the glass or the plastic but on ECM components that either derive from the

serum or are secreted by cells themselves, and that are firmly sticking to the glass of the plastic. Thus, ECM is crucial for cell migration and its properties are known to influence the way cell migrate. Indeed, signaling from ECM receptors helps the cells to interpret its surrounding as the matrix can vary in rigidity, density, topology as well as biochemical composition. In response to the matrix properties, the cell adapts its phenotype. For example, ECM elasticity influences stem cell lineage differentiation (Engler et al. 2006) and modulates cell proliferation, survival and migration (Wells 2008; Zaman et al. 2006). The ECM also regulates the activation and availability of many secreted proteins that diffuse in the extracellular space. In fact, by binding and delivering growth factors, the ECM also controls cell proliferation, survival and migration. Finally, an important factor to take into account is the dimensionality of the ECM. While most studies have long been performed on 2D substrates coated with ECM components, the ECM in which many cells migrate in vivo actually forms a 3D environment. This is a major difference that is now acknowledged to deeply impact the way cells migrate.

I.4.1 - Composition and structure

The matrix composition and organization vary from one tissue to another. Its most abundant components are glycosaminoglycans (GAG), proteoglycans (PG) and fibrillar proteins (Frantz, Stewart, and Weaver 2010; Hynes and Naba 2012; Naba et al. 2012) that are notably secreted by fibroblasts (Green and Goldberg 1964). Nevertheless, other proteins can be found within the ECM. These proteins regulate both the matrix (eg. metalloproteases) and associated cells (eg. growth factors). Fast matrix protein turnover and constant remodeling make the matrix a very dynamic structure. The ECM can be organized into different structures such as the basement membrane, a very thin (100 nm) and dense ECM sheet that supports epithelia, or as a 3D scaffold supporting connective tissues. I will here only describe the composition and organization of these latter form of organization as this is the one that supports the migration of cancer cells used during my PhD.

I.4.1.1 - Glycosaminoglycans and proteoglycans

GAGs are repetition of disaccharides and PG (eg. decorin) are glycoproteins form by the association of a protein chain and a glucidic chain (GAG). Most of ECM space is occupied by PG (Järveläinen et al. 2009). These structures are very hydrophilic and participate to the

formation of a hydrate gel that help the cells resisting compression and strengthening (Mouw, Ou, and Weaver 2014). They also bind to fibrillar components of the ECM. A very common GAG is heparan sulfate (HS) that form heparan sulfate proteoglycans (HSPG) that can be both purely extracellular (eg. perlecan or collagen XVIII) or membrane-bound (eg. syndecan). HSPG are known to bind growth factors and thus play an important role in gradient formation. HSPG-bound growth factors are protected from proteolysis and HSPGs also regulates their diffusion through degradation of the HS chains (Sarrazin, Lamanna, and Esko 2011).

I.4.1.2 - Fibrillar proteins

Among fibrillar proteins found in the ECM, there are large glycoproteins with a structural role (eg. elastin and collagen) and non-collagenic glycoproteins that mostly bring resistance to the ECM (eg. laminin and fibronectin). All these ECM proteins binds to each other to form a deeply interconnected network. In addition, collagens can also be crosslinked into more or less dense networks (Hynes and Naba 2012). Once secreted, the precursor tropoelastin assembles into fibers before to be covered with glycoproteins microfibrils (Wise and Weiss 2009). Laminin is a heterotrimeric protein that self-assemble into a cross-linked web and is critical component of basement membranes. Fibronectin helps to organize the ECM and plays a major role in cell adhesion. It is secreted by many cell types. It can help cells to sense ECM tension as cryptic integrin binding sites are uncovered in the fibronectin structures when it stretches (Smith et al. 2007). Fibronectin is first deposited on an existing ECM network before to be reorganized into a dense interconnected network (Chernousov et al. 1991).

I.4.2 - Collagen type I

The collagen superfamily comprises 28 members in vertebrates and collagens are the most abundant proteins in our body. Collagens act as structural scaffolds that can also bind ECM partners and growth factors. Type I collagen is ubiquitous but other collagen types expression can be restricted to specific tissues. Type I collagen is produced by fibroblasts, endothelial and epithelial cells (Bosman and Stamenkovic 2003). It is the major constituent of the stroma in which cancer cells escaping from the primary tumors have to migrate.

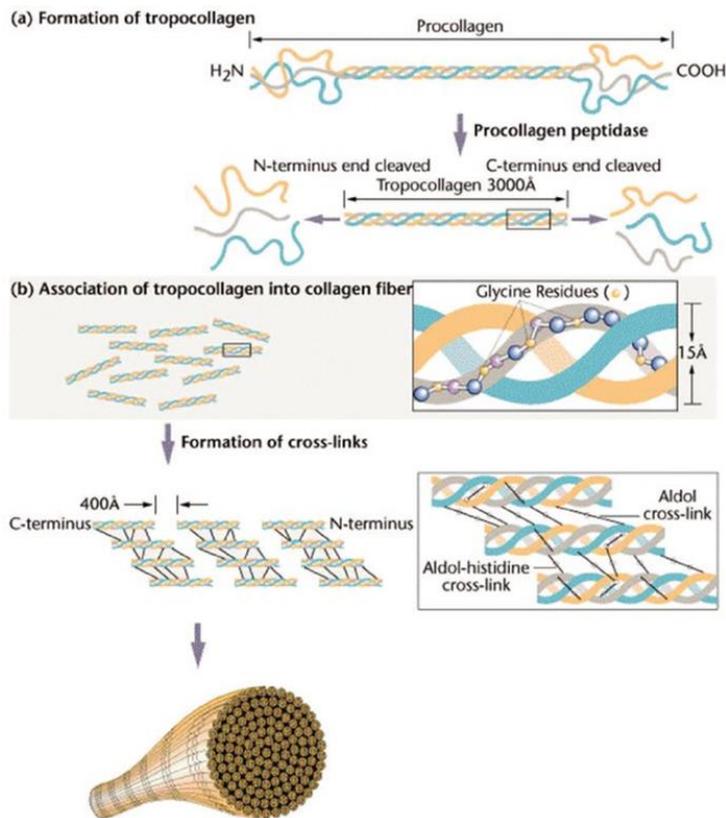


Figure 16. Collagen fiber formation starts with the secretion of tropocollagen, a triple helical protein. Upon cleavage of both its extremities, tropocollagen can assemble into microfibrils. These microfibrils can then assemble into fibers that can be up to 500 nm wide and 1 cm long (Bhattacharjee and Bansal, 2005)

I.4.2.1 - Collagen structure and fibrillogenesis

Type I collagen expression is regulated by EGF and Integrin $\alpha 2\beta 1$ activation (Creely et al. 1990; Ivaska et al. 1999). Collagen is secreted as a triple helical protein called tropocollagen. It is composed of three α chains that can be identical or not. Chains contains Gly-X-Y repetition, X and Y often being proline and hydroxyproline. Presence of proline favors chain folding and hydroxyproline makes hydrogen bond between chains. Tropocollagens presents a 300 nm long and 2 nm wide triple helix structure that can further establish covalent bounds between them to form microfibrils. These microfibrils can then assemble into fibers that can be up to 500 nm wide and 1 cm long (Craig et al. 1989) (Figure 16). The process of fibrillogenesis stabilizes the triple helix of each tropocollagen subunits, providing a strong resistance to tensile forces (Buehler 2006). The process of fibrillogenesis is autonomous and do not require the assistance of cells. Consequently, Type I collagen I can conveniently polymerize and form a network in vitro (Gross and Kirk 1958; Wood and Keech 1960). The fibers diameter and network density vary with the polymerization conditions and particularly with the temperature (Doyle et al. 2015; Figure 17). In vivo, fibroblasts can further remodeled collagen fibers into bigger structures like sheets and cables.

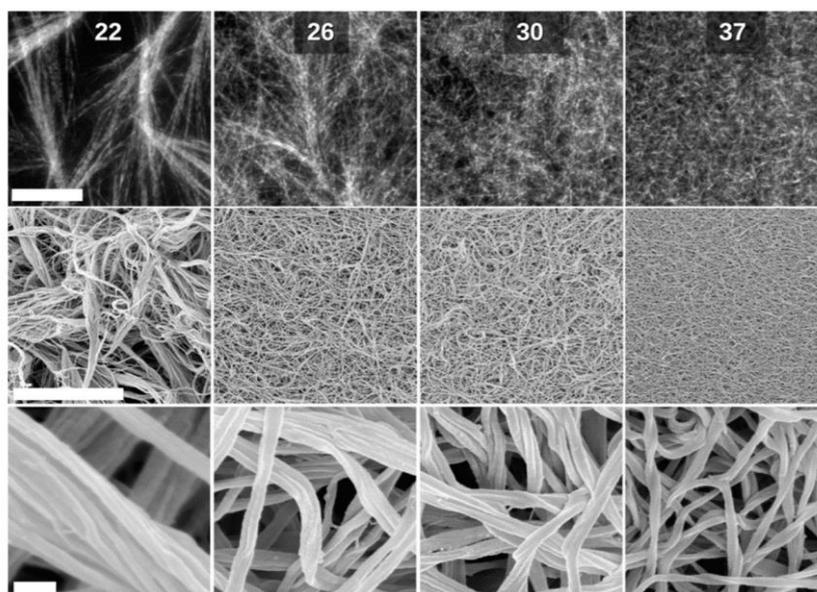


Figure 17. Effect of the temperature (22/26/30/37°C) on collagen network polymerization *in vitro*. Row 1 show confocal reflection images and row 2/3 show SEM images at different magnification. The scale bars represent 20 μm (rows 1 and 2) and 200 nm (row 3) (From Jansen et al., 2018)

I.4.2.2 - Collagen binding proteins

Type I collagen can interact with many different proteins (Figure 18). It first interacts with its receptors and notably integrins. One microfibrils exposes on integrin binding site, allowing the clustering of integrins on collagen fibers (Sweeney et al. 2008). Numerous GAGs also bind to collagens to form a complex matrix. Many matrix metalloproteases (MMPs) bind

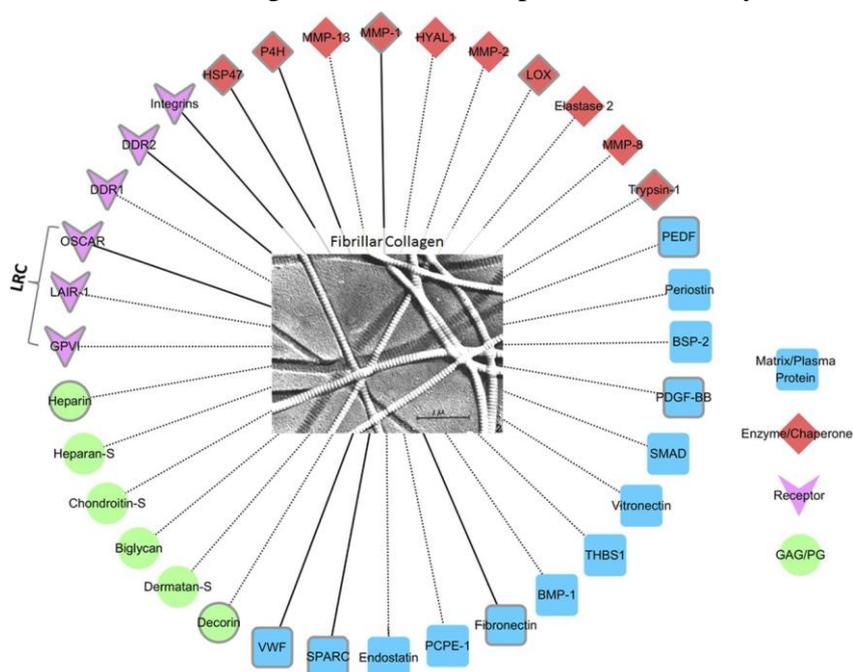


Figure 18. Fibrillar collagen can interact with many proteins including its receptors at the cell membrane, GAG and PG, other fibrillar proteins form the matrix, soluble growth factors diffusing in the extracellular matrix and the different collagenases. (From Ann and Brodsky, 2016)

and cut collagen fibers. Such cleavages can expose RGD sites in the collagen structure that can then be bound by $\alpha\beta_3$ integrins (Petitclerc et al. 1999). Factors that are only found in the blood stream such as fibrin (Reyhani et al. 2014), the von Willebrand factor (Pietu et al. 1987) and the Platelet Collagen Receptor Glycoprotein VI (Miura et al. 2002) also bind to collagen. Collagen is only exposed to blood in case of vessel breakage and in that case these proteins help the binding of platelet to collagen and thus the clot formation (Farndale et al. 2004). Fibronectin binding to collagen (Pearlstein 1976) can guide migration through haptotaxis (Attieh et al. 2017). Collagen also binds growth factors like hepatocyte growth factor (HGF) (Schuppan et al. 1998) and transforming growth factor β (TGF- β) (Paralkar, Vukicevic, and Reddi 1991).

I.4.3 - Growth factors and the ECM

Growth factors are secreted proteins that regulate cell functions such as apoptosis, cell cycle progression, differentiation and migration. The ECM can modulate the activity of growth factors in many ways. First, the ECM regulates the availability of many growth factors. Indeed, binding of growth factors to the ECM modulates their diffusion rate and protects them from proteolysis. Thus, ECM can be seen as reservoir for growth factors. ECM also regulates growth factors spatial distribution. This regulation is key to establish gradients that are necessary for wound healing or morphogenesis (Kreuger et al. 2004). Because the ECM binds to both integrins and growth factors, it creates local formation of signaling complexes (Rahman et al. 2005). In addition, ECM-bound growth factors can display delayed endocytosis and degradation that could lead to more sustained signaling (Platt et al. 2009).

MMPs play a major role in regulating growth factors activity in the ECM. MMPs free peptides trapped in the ECM (Imai et al. 1997; Suzuki et al. 1997). Proteases can also cleave growth factors, leading to their activation (Lyons et al. 1990; Naldini et al. 1992). In addition, laminin and tenascin cleavage releases EGF-like domains that can activate the EGF receptor (Schenk et al. 2003).

I.4.3.1 - Growth factors binding the ECM

Many growth factors bind to the ECM. Fibroblast growth factor (FGF) binding to HSPG is involved in neural development (Nurcombe et al. 1993). Vascular endothelial growth factor (VEGF) binding to the ECM plays a role in angiogenesis (Gerhardt et al. 2003). ECM-associated hepatocyte growth factor (HGF) regulates mammary gland ductal branching (Garner et al. 2011) and heparin-binding EGF-like growth factor (HB-EGF) stimulates smooth muscle cells migration (Higashiyama, Abraham, and Klagsbrun 1993). HGF binds to fibronectin and vitronectin. This leads to c-Met and integrins forming signaling complexes (Rahman et al. 2005). Tumor necrosis factor α (TNF- α) binding to fibronectin is involved in clot formation (Alon et al. 1994). VEGF binding to fibronectin and tenascin promotes cell proliferation (Ishitsuka et al. 2009; Wijelath et al. 2006).

Much less growth factors are known to bind to collagen type I. Platelet-derived growth factor (PDGF) and HGF bind to collagen type I and this modulates their bioavailability (Schuppan et al. 1998; Somasundaram and Schuppan 1996). Collagen-bound HGF maintain its activity. Collagen type I also binds to TGF- β (Paralkar, Vukicevic, and Reddi 1991).

In addition, many research efforts are put in the creation of fusion proteins associating growth factors and ECM-binding domains to generate clinically useful growth factors-containing matrix scaffolds. Applied to a wound, these medicines would be more stable and less diffusive and show some clinical advantages.

I.4.3.2 - Growth factor gradients *in vivo*

Gradients of growth factors have been observed in several model organism. In the *Drosophila* egg chamber, border cells use EGF receptor and polio virus receptor (PVR) to follow TGF- α and PDGF- and VEGF-related factor 1 (PV1) gradients (Duchek et al. 2001; Duchek and Rørth 2001). Bone morphogenetic protein (BMP) gradients drive lateral cells positioning during zebrafish gastrulation (von der Hardt et al. 2007). Heparan-bound VEGF gradients guide endothelial cells migration-dependent vascular sprouting during mice retina formation (Gerhardt et al. 2003). Heparan-bound CCL21 gradients in mouse skin drive dendritic cell haptotaxis toward lymphatic vessel (Weber et al. 2013).

I.4.4 - 2D versus 3D ECM and consequences for cell migration

For many years, cell migration has been studied in the Petri dish, i.e. on 2D glass or plastic. However, *in vivo*, cells often migrate in a 3D ECM whose mechanical properties are completely different from the ones of glass or plastic. More and more, investigators focus on cell migration in physiologically relevant, 3D matrices reconstituted *in vitro* or directly *in vivo*. These studies have highlighted the profound differences between cell migration on 2D versus in 3D substrates (Figure 19A). A popular model is the *in vitro* reconstituted 3D type I collagen matrices that are easy to generate and reproduce the characteristics of cell migration in the stroma. An obvious differences between 2D and 3D is that cells evolving in a 3D network composed of collagen fibers need to squeeze between the pores formed by the network, and, in the case of mesenchymal-like migration, also need to degrade fibers in order to open a way for migration. Another fundamental difference is the way cells adhere to their environment as both the topology and the mechanical properties of 3D networks are completely different from the ones found on 2D, rigid substrates.

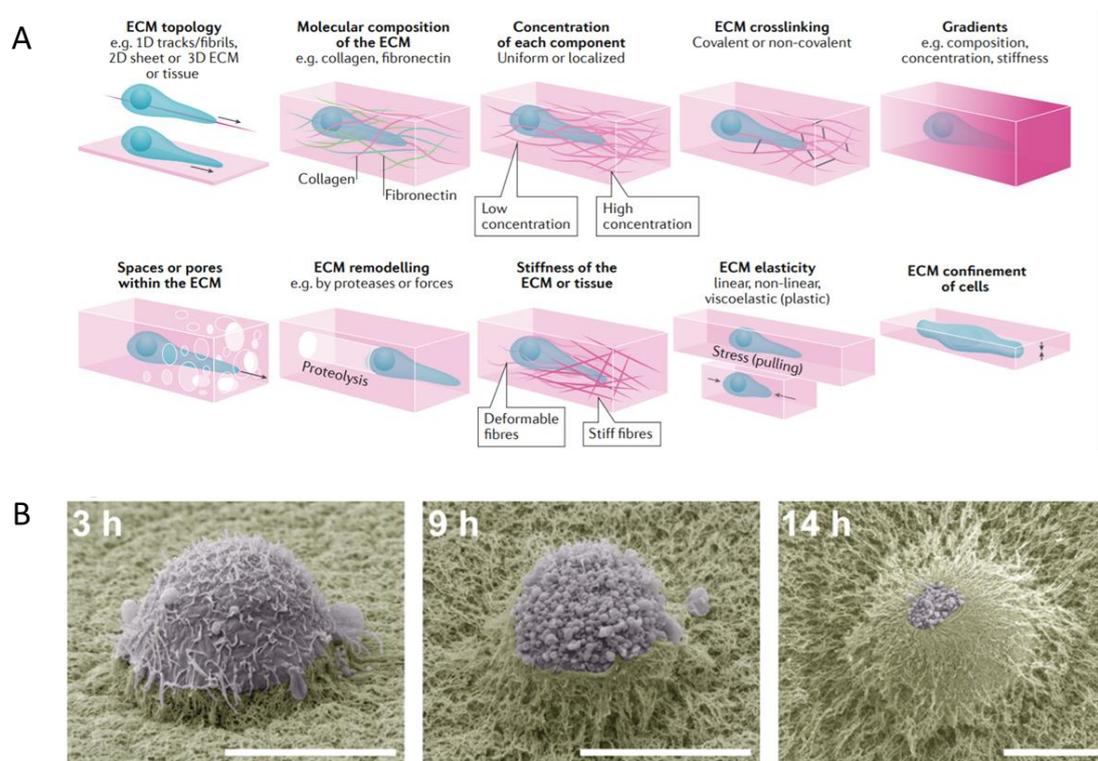


Figure 19. A) Matrix properties regulate 3D cell migration, cell migration is affected by the matrix composition, the concentration of its component, the level of matrix reticulation, gradient of various factors, stiffness gradient, matrix density, the cell ability to degrade or apply forces to the matrix, the matrix elasticity (From Yamada and Sixt, 2019) B) To enter a 3D network, a migrating cell needs to progressively remodel the matrix around it. SEM images of MDA-MB-231 (violet) entering Matrigel (green), a mix of matrix proteins. Scale bars, 10 μm (Poincloux et al., 2011).

I.4.4.1 - Matrix degradation

In order to move in dense 3D environments, cells need to clear the way in front of them (Figure 19B). To do so, cells can secrete metalloproteases that degrade the surrounding matrix. Some cells that efficiently move in 2D cannot move in 3D gels because they do not express proteases. There are two main metalloprotease families (P. Lu et al. 2011), the matrix metalloproteinases (MMPs) that mostly degrade fibrillar proteins and the ADAMS (A Disintegrin And Metalloproteinase with Thrombospondin Motifs) that mostly degrade proteoglycans.

There are 23 MMPs in human. MMP-1 (or collagenase I), MMP-8 and MMP-13 are the three secreted collagenases. Membrane-type 1 MMP (MT1-MMP) is a transmembrane MMP that degrades type I collagen (Sabeh et al. 2004). MMPs expression can be induced by many growth factors including EGF (Kajanne et al. 2007). In addition collagen induces MMPs expression through DDR signaling (Vogel et al. 1997). Like many proteases, MMPs are secreted as inactive proenzymes that are then activated, notably by others MMPs. MT1-MMP is targeted to the cell front where it both degrades the matrix and locally activate other MMPs (Mori et al. 2002).

I.4.4.2 - Cell adhesion in 3D

Mechanisms of cell migration have been revealed through studies performed on 2D, rigid environments (glass/plastic). However, *in vivo*, cell migrates in softer 3D matrices. In addition with rigidity and pliability many parameters vary between 2D plastic surface and 3D matrices. Topology in 3D is very different, ligands are not presented evenly distributed as on planar surfaces but tightly clustered on fibers. In addition, the elasticity of a 3D collagen network is much smaller than the one of glass or plastic (approximately 200 Pascals versus 1 gigaPascals).

Substrate rigidity positively regulates FAs size and stress fibers formation. Thus, it is not surprising that the existence of these structures is less easy to spot in 3D matrices (Burrige et al. 1988; Fraley et al. 2010), even if they have been observed (Cukierman et al. 2001; Kubow and Horwitz 2011; Figure 20). In 3D, FAs are smaller and stress fibers are less aligned with the major cell axis of the cell (Discher, Janmey, and Wang 2005; Prager-Khoutorsky et al. 2011).

FAs composition changes in 3D. In a landmark study, vinculin and FAK were confirmed to be present at FAs in 3D networks, but FAK phosphorylation levels were shown to be much weaker than on 2D. On the opposite, $\alpha 5$ integrins and paxillin colocalize in 3D but not in 2D (Cukierman et al. 2001). 3D FA components have faster turnover than their 2D counterparts (Doyle et al. 2012). As a conclusion, molecular players remain the same in 3D but organization, dynamics and phosphorylation state vary.

We recently reported that cancer cells migrating in a 3D network use another type of adhesive structures, in addition to FAs. We observed that collagen fibers pressing on the plasma membrane generates a local membrane curvature that is a signal for local nucleation of clathrin-coated structures (CCSs) (Elkhatib et al. 2017). These particular CCSs are enriched in $\beta 1$ -integrin, a collagen receptor, and adopt the rod-like shape of fibers by forming tubular structures. These so-called tubular clathrin/AP-2 lattices (TCALs) actually wrap around fibers as they try and fail to internalize them. As a consequence, TCALs pinch collagen fibers and provide new anchoring points to the 3D substrate. This helps the cell to stabilize long protrusions by reducing the tension that raises across the protrusion as it grows. Thus, the particular topology of 3D collagen networks allows the formation of a new class of adhesion structures that fully participates in the migration process (see more details in the second chapter of this manuscript).

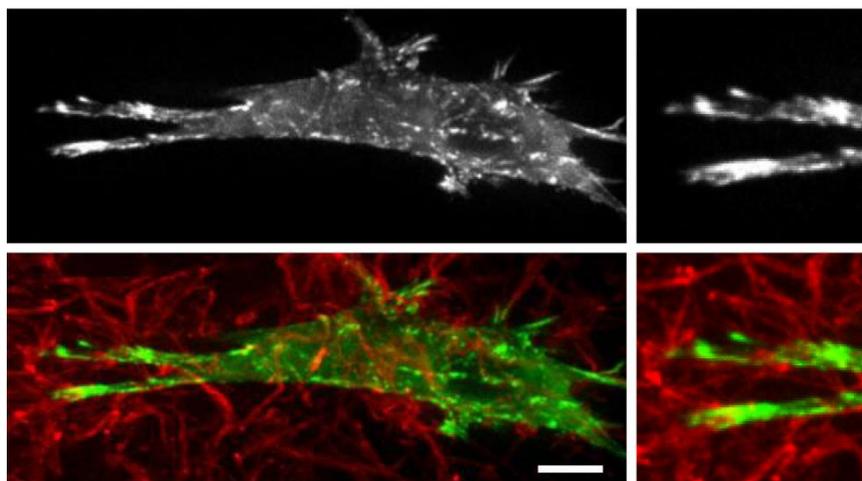


Figure 20. Cells migrating in 3D also show focal adhesions at the leading edge. A human fibroblast expressing EGFP-talin migrates inside an *in vitro* polymerized collagen network and seems to use focal adhesions similarly as described in 2D migration. Scale bars: 10 μ m (Doyle and Yamada, 2015)

II - CLATHRIN-MEDIATED ENDOCYTOSIS

Endocytosis is a fundamental process that allows the cell to acquire some elements from its environments. It relies on the formation of plasma membrane invaginations that surround the material to be internalized. It is an active process requiring energy and that culminates in the detachment of the invagination from the plasma membrane as it buds inside the cytosol. The first described endocytosis process was phagocytosis (from the Greek “cell eating”). Besides phagocytosis, a number of pinocytosis (from the Greek “cell drinking”) pathways have then been described. The major function of these pinocytosis pathways is actually not to internalize extracellular fluids but rather to control the uptake of cell-surface receptors and their ligands.

Clathrin-mediated endocytosis (CME) is the major and best described pinocytosis pathway and is used by all eukaryotic cells. It happens during the whole cell life with a notable decrease during mitosis (Warren, Davoust, and Cockcroft 1984). During CME, cells internalize membrane portions and their associated receptors or cargoes (eg. GPCR, RTKs, integrins and ions channels). These receptors can be internalized with or without their ligands. They are internalized into vesicles that further traffic inside the cytoplasm.

Because of the large range of receptors it internalizes, CME is key to many cellular processes. CME helps to regulate the membrane composition in time and space by removing some of its components. By controlling the composition of the membrane, it regulates most of the cell’s interaction with its environment. It participates to the sampling of the environment. It regulates nutrient uptake. CME also controls signaling pathways through regulating endocytosis of signaling receptors. CME plays a major role in cell migration, notably through FAs turnover and polarized redistribution of receptors.

II.1 - CLATHRIN-COATED STRUCTURES

CME relies on the formation of clathrin-coated structures (CCSs) at the internal leaflet of the plasma membrane. CCSs formation is a multistep process starting with the nucleation of these structures at the plasma membrane followed by cargo selection, coat maturation, scission and uncoating (McMahon and Boucrot 2011). During the nucleation, a small core of proteins including clathrin and its adaptor AP-2 are recruited at the plasma membrane and defines the

endocytic site. Cargoes (i.e. receptors) are recruited by adaptor proteins and accumulate within the endocytic site that concomitantly starts to invaginate. At this stage, CCSs are often referred to as “clathrin-coated pits” because of the particular shape of this structures as seen in electron microscopy pictures. The continuous invagination process then leads to the formation of a vesicle that is only linked to the plasma membrane by a thin neck. Dynamin-mediated scission of this neck frees the vesicle in the cytosol. Once in the cytosol, the different constituents of the clathrin coat disassemble, and the vesicle starts to traffic inside the cell. From then, receptors will be either degraded in lysosomes or be recycled back to the plasma membrane (Figure 21).

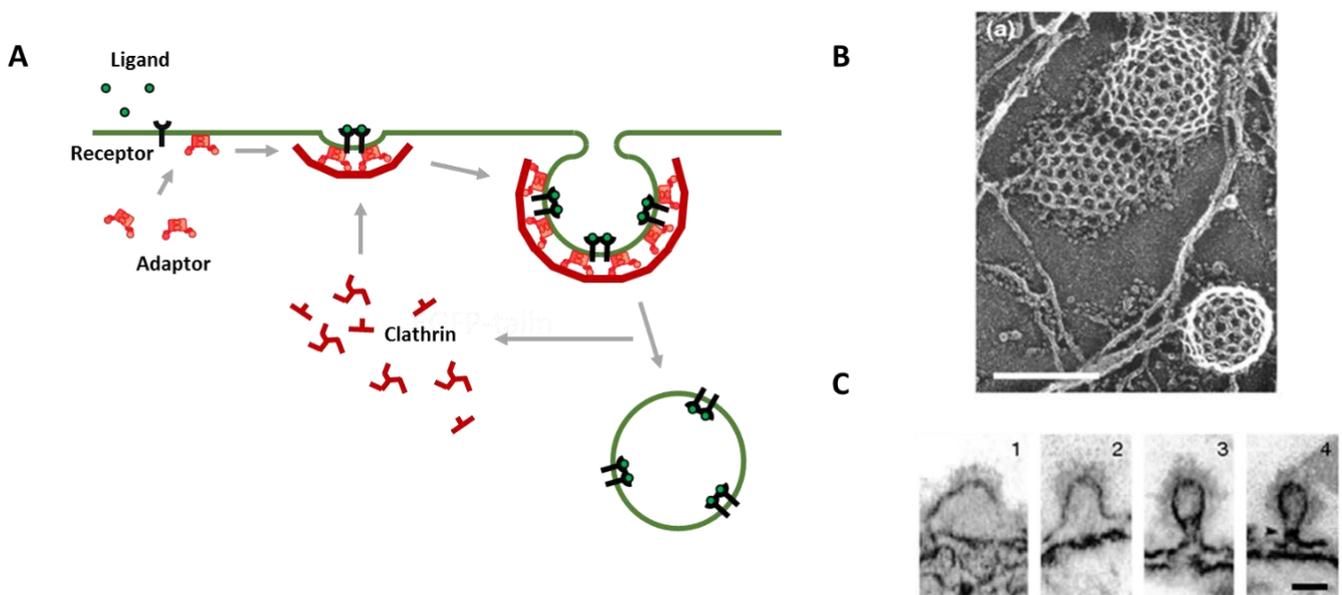


Figure 21. A) CME initiates with the binding of adaptor proteins to membrane receptors. Then clathrin is recruited and polymerizes into a coat. The clathrin coated region invaginates progressively to form a vesicle that finally separates from the plasma membrane and traffics inside the cell. The protein coat disassembles and is available for a new cycle. B) EM image of a carbon platinum replica showing different steps of CME in a fibroblast. Scale bar = 200 nm. C) EM images of cryosliced neuron showing different steps of CME. Scale Bar = 200 nm (Higgins and McMahon, 2002).

II.1.1 - Main actors of CCSs

If CME is named after clathrin, over 50 other cytosolic proteins are involved in the formation and dynamics of CCSs. Interactions between these proteins are highly organized, and the regulation of their assembly drives the maturation of a CCSs into a vesicle (Figure 22). The BAR proteins FCHO1,2 play a key role during nucleation. Adaptor Protein 2 (AP-2) is central

to cargo selection and recruitment. Clathrin drives the coat assembly. Dynamin is required for scission and auxilin for the coat disassembly. Because of their critical role and the numerous interaction clathrin and AP-2 establish at CCSs, they have been highlights as central hubs of CCSs in the literature (Schmid and McMahon 2007).

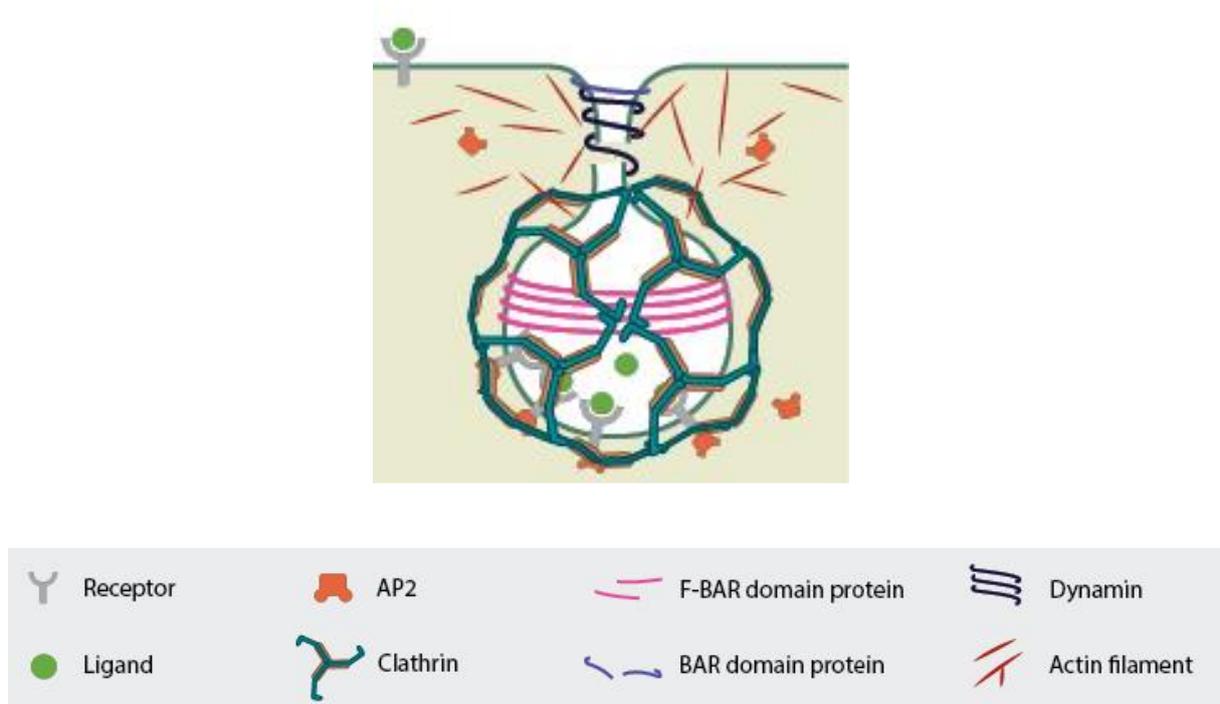


Figure 22. The main actors of clathrin-mediated endocytosis. BAR proteins play a key role during nucleation and membrane shaping. Adaptor Protein 2 (AP-2) is central to cargo selection and recruitment. Clathrin drives the coat assembly. Dynamin is required for vesicle scission. (From <https://www.mechanobio.info>)

II.1.1.1 - Adaptor protein 2

Adaptor proteins (APs) are a family of heterotetrameric complexes comprising AP1, AP2, AP3 and AP4 that are all involved in membrane budding events inside the cell. Their primary function is to bridge clathrin to membranes and to recruit receptors destined to internalization. AP2 is only found at the plasma membrane whereas AP1, AP-3 and AP-4 are found at endosomes and the Golgi apparatus. AP-2 is a heterotetramer consisting of four subunits: α -, β 2-, μ 2- and σ 2-adaptins (Figure 23A). Adaptins assemble to form the core structure of the AP-2 complex with two appendages connected by flexible linkers. 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 flexible domain of β 2-adaptin is essential to interact with clathrin (Shih, Gallusser, and Kirchhausen 1995) and the appendage domains of both α - and β 2-adaptins are involved in interactions with other adaptors and accessory proteins that participate in CCSs formation and dynamics. α -adaptin trunk domain binds to phosphatidylinositol-(4,5)-biphosphate (PIP2), a phospholipid specifically found at the plasma membrane, and thus provides anchorage to and specificity for the plasma membrane. μ 2-adaptin and a structural domain formed by α 2- and σ -adaptins are responsible for direct interaction with receptors through their tyrosine-based Yxx Φ (with Φ being a bulky hydrophobic amino-acid) or dileucine-based (D/E)xxxL(L/I) internalization motifs, respectively. Phosphorylation of μ 2-adaptin induces a conformational change that increases AP-2 affinity for both cargos and PIP2 (Höning et al. 2005). The conformational changes occur after initial, weak interaction with PIP2 at the plasma membrane and is thus crucial to ensure early stages of CCSs assembly. It has been suggested that AP-2 may not be necessary for the formation of all CCSs in particular experimental conditions. However, in physiological conditions, AP-2 is present in all CCSs and its depletion by siRNAs leads to an almost complete depletion of CCSs. Because of the many interactions it has with other CCSs components and its prominent role in CCSs formation, AP-2 is considered as the central hub of CCSs (Conner and Schmid 2003; Schmid and McMahon 2007).

II.1.1.2 - Clathrin

Clathrin doesn't directly bind to membranes and thus needs adaptors to be recruited at endocytic sites. Clathrin assembles into a hexamer constituted by three clathrin heavy chains (CHC, 190 Kda) each associated with a clathrin light chain (CLC, 30 Kda) (S. H. Liu et al. 1995; Ungewickell and Ungewickell 1991). The heavy and light chains assemble into a triskelion that can polymerize by themselves into a basket-like cage *in vitro*, in particular buffer conditions (Crowther and Pearse 1981; Keen, Willingham, and Pastan 1979; Ungewickell and Branton 1981; Figure 23B). *In vivo*, adaptors-assisted polymerization of clathrin triskelia on membranes forms a honey comb-like structure that is constituted either only of hexagons when the clathrin coat is flat, or of a mixture of hexagons and pentagons when the clathrin-coat is curved (Fotin et al. 2004, 2006). The role of clathrin has long been seen as a driver of membrane curvature generation and indeed, *in vitro* studies suggest that it could fulfil this function (Dannhauser and Ungewickell 2012). However, many other proteins of CCSs play a role in

membrane curvature and clathrin may only be there to stabilize this curved state (B. L. Scott et al. 2018).

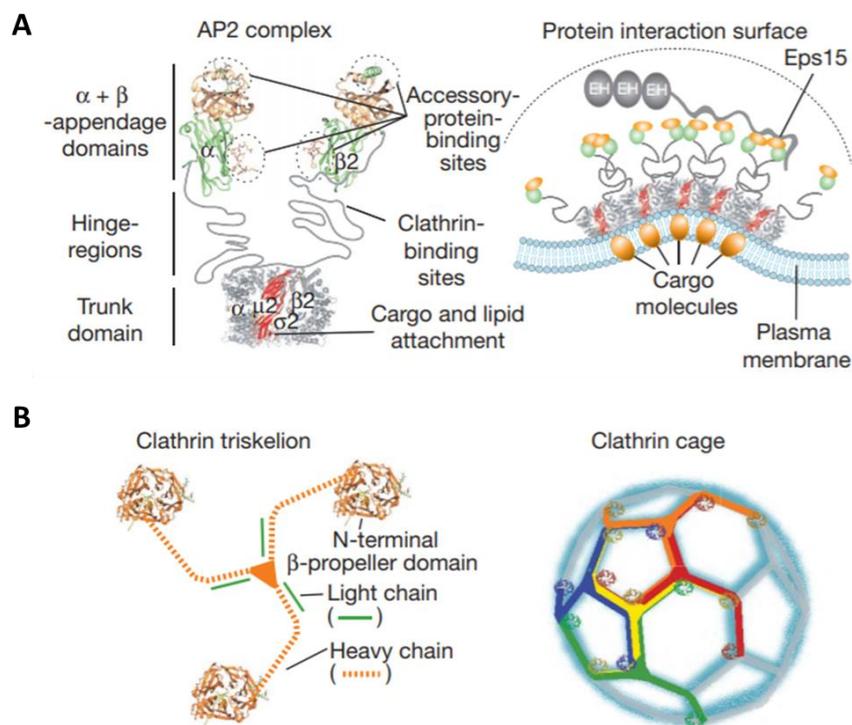


Figure 23. A) AP-2 consists of four subunits: α -, β 2-, μ 2- and σ 2-adaptins. These subunits mediate interaction with most of the actors of CME. AP-2 is a key actor of CME as it bridges together the plasma membrane, clathrin and their cargoes. B) Clathrin assembles into a hexamer of three clathrin heavy chains associated with three clathrin light chains. The heavy and light chains assemble into a triskelion that can polymerize by themselves into a basket-like cage in vitro.

II.1.1.3 - Accessory proteins

In addition to clathrin and AP-2, many other proteins participate in the formation and dynamics of CCSs. Most of them can bind to both PIP2 and AP-2 and/or clathrin. These proteins are involved in cargo selection, membrane bending, clathrin recruitment and assembly, scission or uncoating. The functions of these accessory proteins are highly regulated as they are recruited at very precise stages of CCSs maturation and at very precise substructural locations (Mettlen et al. 2018; Taylor, Perrais, and Merrifield 2011). Below, I will mention and discuss some of these proteins in further details when describing the different steps of CCSs dynamics.

II.1.2 - Life cycle of CCSs

Canonical CCSs are small (approximately 100 nm in diameter), dynamic structures whose life cycle is precisely regulated. This cycle starts with nucleation, followed by a maturation step that corresponds to membrane bending and vesicle formation, and then a scission step that allows the physical separation of the nascent vesicle from the plasma membrane, and finally an uncoating step that removes the clathrin and adaptor coat from the vesicle. Depending on the cell type, this cycle can last between 30s and 1 min approximately.

II.1.2.1 - CCS nucleation

CCSs formation starts with the recruitment of core endocytic proteins that progressively recruit the entire coat. A random walk of these core proteins on the internal leaflet of the plasma membrane was proposed to lead to CCSs formation at random positions at the plasma membrane (Ehrlich et al. 2004). Yet, the precise molecular time course of CCSs nucleation is still a matter of debate (Cocucci et al. 2012; Henne et al. 2010). Both AP-2 and the BAR domain protein FCHo1/2 have been proposed as key regulators of CCS initiation. AP-2 was the first proposed master regulator of initiation, notably because of its interaction with numerous endocytic proteins. As it binds both clathrin and PIP2, it is key to clathrin recruitment at the plasma membrane. AP-2 is also one of the earliest arriving protein at the endocytic site (Cocucci et al. 2012). Nevertheless some studies suggested that AP2 depletion doesn't completely abolish the formation of CCSs (Aguet et al. 2013; Motley et al. 2003) but this may depend on experimental conditions. In 2010, Henne and colleague proposed that FCHo1/2 is the main regulator of CCSs nucleation through generating an initial, shallow membrane curvature but this model has been challenged (Cocucci et al. 2012). Finally, PIP2 is also known to play a major role in CME initiation (Jost et al. 1998). It helps to the recruitment of several CCSs proteins at the plasma membrane and its depletion inhibits CCSs formation (Antonescu et al. 2011)

Receptors themselves have been proposed to be able to control the *de novo* formation of CCSs upon their activation (Wilde et al. 1999). However, most studies suggest that activated receptors are actually targeted to preexisting CCSs in order to be internalized (Rappoport and Simon 2009; Scott et al. 2002). Yet, it is also now clear that cargo recruitment affects CCS dynamics and stabilizes them upon nucleation (Henry et al. 2012). In addition, receptor

overexpression, or experimentally-induced clustering, leads to an increased CCSs nucleation (Liu et al. 2010; Mettlen et al. 2010). Some viruses are also able to nucleate their own CCSs in order to be internalized (Rust et al. 2004), but others were shown to reach preformed CCSs (Ehrlich et al. 2004). Finally, we recently demonstrated that collagen fibers contacting the plasma membrane induce local CCSs nucleation (Elkhatib et al., 2017). This is most likely due to collagen fiber inducing a local plasma membrane curvature that is probably a signal triggering local CCSs nucleation. In conclusion, CCS nucleation causes are not completely understood, and this probably reflects variability and redundancy in the nucleation mechanisms.

II.1.2.2 - CCS maturation

Upon nucleation, CCSs may not necessarily pursue to a full endocytosis process and, indeed, studies have suggested the existence of an “endocytic checkpoint” determined by the coat composition and especially receptors recruitment (Aguet et al. 2013). If CCSs do not contain enough cargos, maturation is delayed and, in some cases, the coat disassembles without proceeding to endocytosis (Loerke et al. 2009). This allows to prevent the formation of “empty” endocytic vesicles.

A wide array of receptors are internalized through CCSs. Receptor’s recruitment can either be constitutive (i.e. no need for ligand binding) or require ligand-mediated activation of the receptor. Nutrient receptors are mainly internalized constitutively whereas the internalization of signaling receptors usually requires ligand-dependent activation. For example, the transferrin receptor (TfR) is constitutively recruited to CCSs through a direct interaction with AP-2 (Nesterov et al. 1999). On the opposite, the epidermal growth factor receptor (EGFR) recruitment at CCSs only happens upon ligand binding and receptor dimerization and involves multiple endocytosis motifs linking different CCSs proteins (Goh et al. 2010). A full set of adaptors proteins recognize specific motifs in cargos and recruit them to CCSs. Both FCHo1/2 and AP2 can bind to some cargoes and thus, receptors recruitment already happens during the nucleation step (Henne et al. 2010). In addition to AP-2, there are a number of accessory proteins that are dedicated to the recruitment of specific receptors. For instance, β -arrestins are specialized in the recruitment of activated GPCRs (Zhang et al. 1999). Ubiquitin Interacting Motif (UIM)-containing proteins such as Eps15 and epsin can recognize and recruit ubiquitinated receptors to CCSs (Hawryluk et al. 2006). The β integrins domain NPX[FY] and the FDNPVY motif of the low density lipoprotein receptor (LDLR) are recognized by the

clathrin adaptors ARH, Numb and Dab2 via their phosphotyrosine binding (PTB) domains (Maurer and Cooper 2006).

In order to form a vesicle, CCSs need to progressively invaginate. Membrane bending occurs mostly during the maturation step and requires different types of endocytic proteins. Clathrin can form cages in solution (Keen, Willingham, and Pastan 1979) but can also shape membranes into spherical buds *in vitro* (Dannhauser and Ungewickell 2012). It was proposed that progressive clathrin polymerization impose a curvature to the membrane (Kirchhausen and Harrison 1981). However, it was recently shown that the forces produced by clathrin polymerization are not sufficient to account for the observed membrane deformation, especially when membranes are under tension (Saleem et al. 2015). Yet, other endocytic proteins are proposed to increase the stiffness of the clathrin coat, potentially allowing such deformations (Lherbette et al. 2019). *In vivo*, clathrin polymerization synergizes with other mechanisms to produce efficient membrane invagination. Protein with BAR domains also play a role in membrane bending during CME. These proteins display a banana shape that allow them to sense and/or produce membrane curvature (Figure 24A). The F-BAR proteins FCHo1/2 arrives very early at CCSs nucleation sites and only produces a shallow invagination (Henne et al. 2010). N-BAR proteins like amphiphysin and endophilin are more potent curvature inducers but arrive much later and are believed to organize the shape of the neck of still plasma-membrane associated clathrin-coated vesicles (Peter et al. 2004). CLAM/AP180 and epsin are proteins that interact with AP-2, clathrin and PIP2 and that generate curvature through of a N-terminal amphipathic helix in the internal leaflet of the plasma membrane (Miller et al. 2015). Finally, actin was also proposed to push the membrane in order to induce curvature (Figure 24B). Live and electron microscopy imaging confirmed the presence of actin at endocytic site (Collins et al. 2011; Li et al. 2015) but inhibiting the actin machinery revealed that actin play a non-obligatory role during endocytosis (Fujimoto et al. 2000). Actin polymerization actually seems to be required in case of high membrane tension, a mechanical state that tend to oppose budding (Boulant et al. 2011).

To conclude, many of the accessory proteins in charge of recruiting receptors can also bend the plasma membrane making the maturation step the privileged stage of both CCSs invagination and receptor sorting. In fine, all the mentioned mechanisms cooperate to bend the plasma membrane until the formation of a receptor-containing vesicle that is still connected to the plasma membrane through a thin neck.

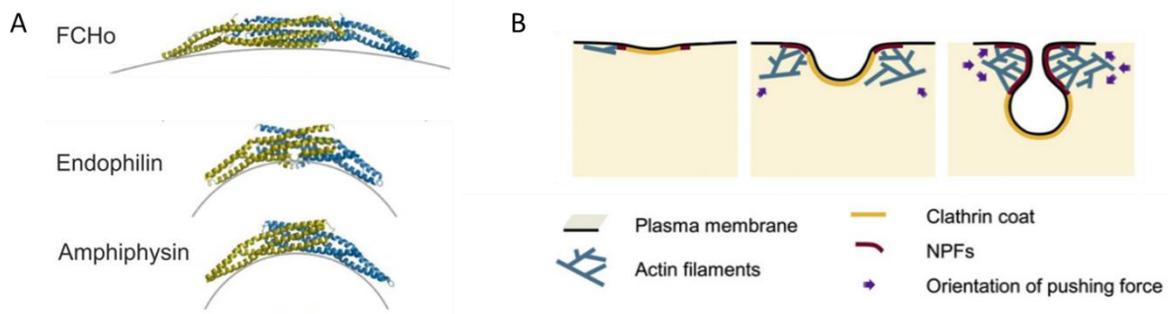


Figure 24. Different mechanisms of membrane bending cooperate to produce the membrane invagination during CME. A) BAR proteins display a banana shape that allows them to sense and/or produce membrane curvature. The different BAR proteins cover a large range of membrane curvature and thus are sequentially recruited to fit the increasing invagination of the membrane. (From Qualmann et al., 2011). B) Actin polymerization has been proposed to participate to membrane bending. It polymerizes against to neck and participates in its constriction. (From Collins et al., 2011)

II.1.2.3 - Vesicle fission

To be released inside the cell, the vesicle needs to break this thin neck. If there is no doubt about dynamin requirement in this process, the exact mechanism of vesicle scission remains unclear. There are 3 different dynamins but only dynamin-2 is ubiquitously expressed. Dynamin-2 is a GTPase of about 100 kDa that assembles into a helix surrounding the vesicle neck (Hinshaw and Schmid 1995). Dynamin-2 is divided into five domains: the PH domain, the GTPase domain, the bundle signaling element (BSE), the stalk domain and the proline rich domain (PRD) (Figure 25A). The PH domain allows membrane anchoring through PIP₂ binding (Zheng et al. 1996). The BSE domain allows the transmission of conformational change between domains. The stalk domain is required for interactions with other dynamin monomers. The PRD interacts with other endocytic proteins.

Dynamin-2 is recruited preferentially to highly invaginated membrane such as the vesicle neck (Roux et al., 2010). Dynamin is also recruited at clathrin structure via the PRD interaction with endocytic proteins such as amphiphysin (Takei et al. 1999). Once at the neck, dynamin-2 interacts with other dynamin monomers through the stalk domain. Dynamin oligomerization progressively forms a helix around the neck. Helix formation enhances the dynamin affinity for GTP (Song, Leonard, and Schmid 2004). Once the helix formed, GTP hydrolysis induces a helical twist that constricts the neck (Antonny et al. 2016; Figure 25B). It is proposed that the helix twist brings the neck membranes close enough to each other so that

they spontaneously fuse due to thermal fluctuation (Bashkirov et al. 2008). Dynamin inhibition completely abolishes CME (Macia et al. 2006) and cells mutated for the GTPase domain display deeply invaginated CCSs with elongated necks (Damke et al. 2001). In addition, actin has been proposed to produce extra forces required for neck constriction during vesicle scission (Collins et al. 2011).

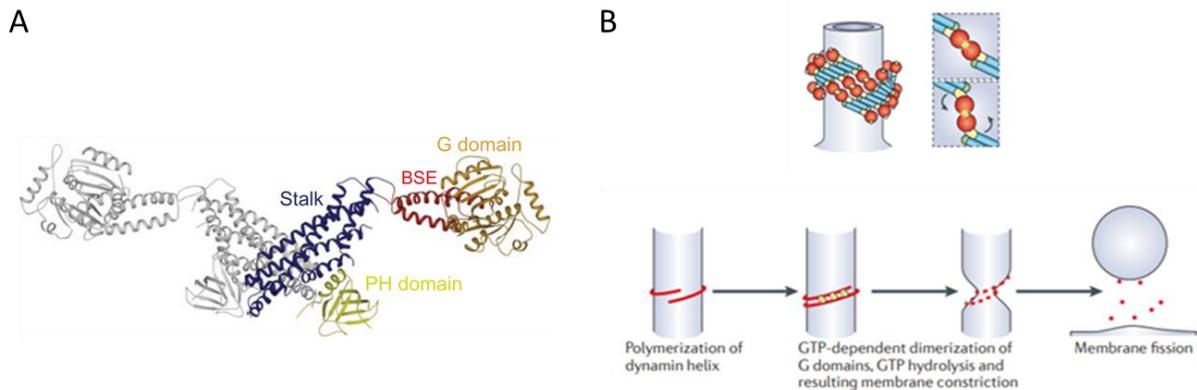


Figure 25. A). 3D structure of dynamin dimers. Dynamin binds the membrane through its pH domain and dimerizes through its stalk domains. B) Dynamin dimers polymerize to form a helix around the vesicle neck. GTP hydrolysis leads to helix constriction and to membrane scission. (From Faelber et al., 2013; Ferguson and De Camilli, 2012)

II.1.2.4 - Coat disassembly

After its scission from the plasma membrane, the newly formed clathrin-coated vesicle needs to get rid of its clathrin and adaptors coat in order to complete endocytosis. This step will allow future inter vesicular fusion events and/or fusion of vesicles with endosomes. Coat disassembly relies on the coordination of two main mechanisms: disruption of the adaptor lattice by PIP2 depletion, and action of the ATPase HSC70 directly on clathrin (Schlossman et al. 1984). Once disassembled, the proteins of the coat can be reused for a new round of CCS formation.

PIP2 dephosphorylation on the vesicle by synaptojanin is involved in the coat disassembly (Cremona et al. 1999). A specific isoform of synaptojanin binds to dynamin and endophilin and is thus recruited at late stages of CME. PIP2 plays a major role in the coat assembly and its disruption destabilizes the coat, especially AP-2. HSC70 is recruited to clathrin-coated vesicles by its co-factor auxilin. Auxilin binds to both clathrin and dynamin and is recruited around the time of vesicle scission. ATPase activity of HSC70 is increased by the

formation of an HSC70-auxilin-clathrin complex. ATP consumption leads to HSC70-controlled modification of the structural arrangement of clathrin triskelia, ultimately leading to clathrin disassembly (Kaksonen and Roux 2018).

Upon uncoating, the endocytic vesicles can fuse with the endosomal system, delivering there the internalized receptors. From there, some receptors can be readdressed to the plasma membrane in a process called recycling, or follow endosomal maturation until being degraded into lysosomes.

II.2 - FUNCTIONS OF CLATHRIN-COATED STRUCTURES

The primary function of CCSs is to regulate the uptake of cell surface receptors and their ligands. By doing so, CCSs allow the cell to control in time and space the composition of the plasma membrane, to acquire nutrients and to regulate signaling pathways. Indeed, many signaling receptors are endocytosed through CCSs and this is known to modulate the quality, strength and duration of the signal elicited by these receptors. Of notes, all of these functions can impact cell migration and indeed, CME has long been recognized to play a major role in cell motility. In additions to these roles that are purely related to their endocytic function, it has recently become more and more clear that at least some subsets of CCSs may play some non-endocytic-related functions. I will here briefly describe the canonical consequences of CME, and especially its role in cell migration, before discussing non-canonical CCSs functions, in particular as signaling platforms and as adhesion structures.

II.2.1 - Canonical consequences of CCSs as endocytic structures

The importance of CME is highlighted by the fact that genetic disruption of this pathway is lethal in most eukaryotes. It is at the heart of the cell program as it regulates directly or indirectly all aspects of cellular functions.

II.2.1.1 - Regulation of plasma membrane composition

The first consequence of CCSs action as endocytic structures is that they endow the cell with the possibility to control the composition of its plasma membrane. Selective endocytosis of some receptors can lead to the depletion of these receptors from the cell surface, or from specific areas of the plasma membrane. For instance, in the case of polarized epithelial cells, CME plays a critical role by maintaining the differential receptor content between the apical and the basolateral side of the cell (Eaton and Martin-Belmonte 2014). Cells round-up when undergoing mitosis and this is achieved by an endocytosis-assisted depletion of adhesion receptors from the plasma membrane. There are many other situations in which CME plays a role by controlling plasma membrane composition and I will below discuss the specific case of cell migration.

II.2.1.2 - Regulation of nutrients acquisition

One way CME and CCSs regulates nutrient acquisition is through controlling the availability of transmembrane nutrient transporters at the cell surface. For example, glucose receptors or the Na⁺/K⁺-pumps are internalized by CCSs (Liu et al. 2004; Wu et al. 2013). But CCSs also directly regulates the acquisition of some non-soluble nutrients like iron and cholesterol (Anderson, Brown, and Goldstein 1977; M. S. Brown and Goldstein 1979; Harding, Heuser, and Stahl 1983; Hentze et al. 2010). The iron carrier transferrin is internalized by CME through binding its receptor TfR. TfR endocytosis is constitutive through directly binding to AP-2. Once in endosomes, the acidic pH leads to iron being released from transferrin. Iron is further transported into the cytosol while TfR/transferrin complexes are recycled towards the plasma membrane. The low density lipoprotein (LDL) is the main carrier for cholesterol. It is composed of cholesterol, phospholipids and a single apolipoprotein B-100 protein. LDL but also VLDL bind to the LDLR in order to be internalized at CCSs. The LDLR receptor binds to apo-B100 and is constitutive internalize through binding to the PTB domains of the clathrin and AP-2 binding proteins ARH or DAB2. Once in the endosomes, the acidic pH lead to LDL being released from LDLR. Endosomes then fuse with lysosomes where the proteins are degraded, and the cholesterol made available for the cell, while LDLR is recycled (Figure 26).

II.2.1.3 - Regulation of signaling pathways

CME plays a major role in signaling and has actually been proposed to be the master regulator of signaling circuits (Scita and Di Fiore 2010). It was first believed for a long time that CME simply shut down signaling pathways by retrieving receptors from the cell surface, preventing them to be exposed to ligands and also to signal from the surface once activated. This vision has been since dramatically challenged and it is now accepted that CME plays a much more complex role in signaling (Vieira, Lamaze, and Schmid 1996).

If internalization of activated receptors prevents their further signaling from the plasma membrane, signaling does not necessarily stop there. Upon endocytosis, activated receptors reach endosomes where their cytoplasmic tails can continue to trigger signaling. Activated receptors spend more time in endosomes than at the plasma membrane. Thus endosomes can also be seen as signaling stations (Grimes et al. 1996). Many receptors like EGFR or TGF β FR remain bound to their ligand within endosomes for a while and thus keep signaling during endosomal trafficking (Haugh et al. 1999; Hayes, Chawla, and Corvera 2002). It was also shown that signaling effectors are recruited at endosomes (Di Guglielmo et al. 1994). It continues during endosomal trafficking until late endosome pH leads to ligand unbinding from its receptor which stops signaling. For the same receptor, signal quality from the plasma membrane can be different from the one emanating from endosomes as some effectors of signaling pathways are only recruited onto endosomal membranes (Hayes, Chawla, and Corvera 2002).

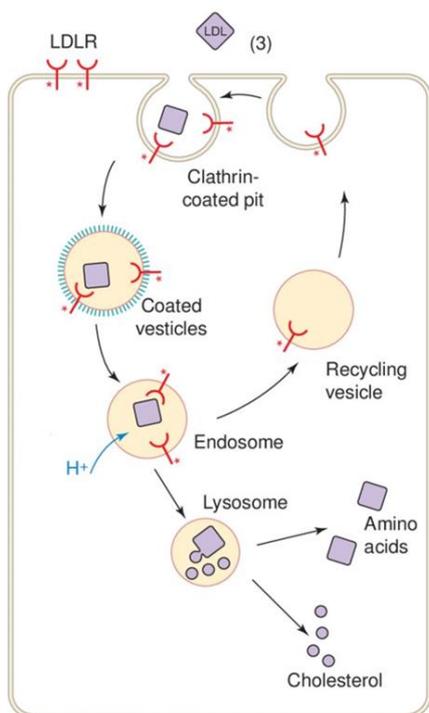


Figure 26. LDL internalization starts with the binding of its apoprotein to the LDLR. LDL/LDLR complex is recruited to and internalized by clathrin structures before to reach endosomes. Once in endosomes, the acidic pH leads to LDL being released from LDLR. Endosomes then fuse with lysosomes where LDL is degraded, and the cholesterol made available for the cell, while LDLR is recycled. (From Beglova and Blacklow, 2005)

II.2.1.4 - CME and cell migration

CME plays a central role in cell migration. Based on 2D studies, it has been proposed that CME could controls endocytosis of adhesion receptors at the rear of the cell, where they are not needed anymore, in order to traffic them towards the cell front where they are the most needed. More generally, endocytosis at the rear and recycling at the front would generate a plasma membrane treadmill mechanism that was proposed to support cell migration (Bretscher 1989). In addition, it was shown that cells can only migrate if using adhesion receptors that can be endocytosed (Bretscher 1992) .

Integrins are continuously internalized (Bretscher 1989; Raub and Kuentzel 1989), mostly through CCSs (Liu et al. 2007), and endocytosis-exocytosis cycles are repeated approximately every 30 minutes for a given integrin (Caswell and Norman 2006). As mentioned above, integrin internalization was first thought to support integrin trafficking from the disassembling adhesions at the retracting rear of the cell to the newly formed adhesions at the protruding front (Lawson and Maxfield 1995; Figure 27A). In addition, CME is now acknowledged to regulate local FAs turnover and signaling as integrin internalization is also observed at the front (Laukaitis et al. 2001; Palecek et al. 1996; Regen and Horwitz 1992; Figure 27B). Yet, some local cycles of internalization at mid-protrusions and recycling at the very leading edge have been observed and thus somewhat support the older model (Caswell et al. 2007). CME also directly regulates the dynamics of FAs, thus indirectly regulating cell migration. Indeed, FAs turnover has been demonstrated to depend on integrin endocytosis at FAs (Ezratty et al. 2009). Most of these studies were based on cells migrating on 2D substrates but CME is also acknowledged to play a critical role in 3D cell migration.

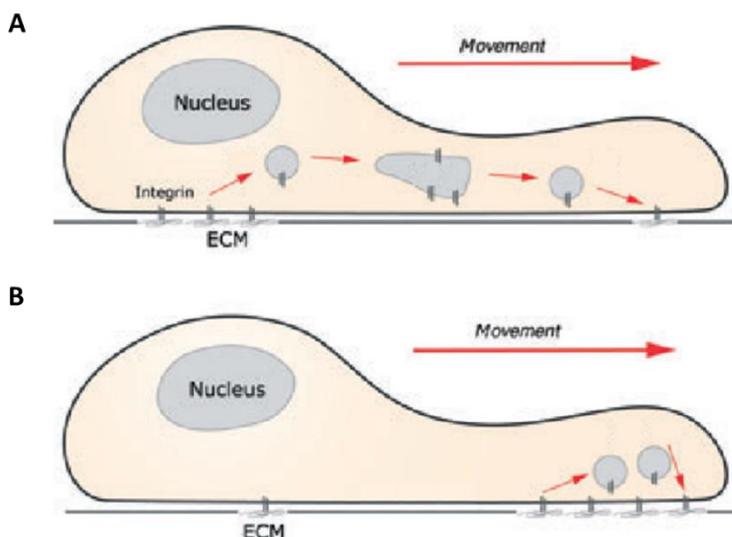


Figure 27. CME brings integrin to the front of migrating cells. A) Integrins are internalized during focal adhesion disassembly at the rear of the cell. Integrins-containing endosomes are routed to the leading edge. B) Focal adhesion turnover and integrin internalization can also be localize at the leading edge. (From Ulrich and Heisenberg, 2009)

CCSs regulate cell migration not only through internalizing integrins but also by regulating signaling pathways. Regulation of the cell-surface level of signaling receptors directly impacts the cell ability to answer to external cues. As already mentioned, activated signaling receptors accumulate into CCSs to be internalized. This limit signaling from the PM itself and, in the case of prolonged exposure to a ligand, it leads to reduced levels of receptors at the membrane. This negative feedback loop prevents excessive signaling. For example, receptor internalization has been shown to be essential during the directed migration of cells following a gradient of growth factor (Maryse Bailly et al. 2000). In addition, perturbing endocytosis results in unbalanced signaling that prevents directed migration (Minina, Reichman-Fried, and Raz 2007). CME can also spatially restricts signaling events in response to extracellular cues. This allows restricted RTKs signaling at the leading edge of *Drosophila* border cells, thus preserving the spatial information of ligand gradients and hence their use for directional migration (Jékely et al. 2005). Finally it is worth saying that growth factors regulate integrin endocytosis and recycling, thus impacting cell migration in another way (Mai et al. 2014)

II.2.2 - Non-canonical functions of CCSs

In addition to the canonical role of CCSs in supporting receptor endocytosis, non-endocytic functions of CCSs have been reported over the years. I will here review some of these atypical functions, in particular the adhesive role of CCSs in cell migration.

II.2.2.1 - Role of CCSs as signaling platforms

CCSs regulate receptor signaling through controlling their endocytosis as mentioned above. But CCSs were also demonstrated to regulate signaling in an endocytosis-independent manner.

First CCSs accumulating at the front of migrating cells have been shown to shape the microtubule network. The growing extremity of microtubules oriented towards the leading edge can occasionally contact some CCSs. The tubulin acetyltransferase α TAT1 is recruited at CCSs through binding to AP-2. When a microtubule contacts a α TAT1-enriched CCS, this leads to the acetylation and stabilization of the microtubule. This mechanism ensures the correct

orientation of microtubules towards the leading edge and is required for efficient persistent migration in a given direction (Montagnac et al. 2013).

It has also become clear that CCSs can serve as signaling platforms for receptor signaling, independently of their endocytic function (Garay et al. 2015). Along this line, prolonged CCSs lifetime was associated with stronger signaling (Eichel, Jullié, and von Zastrow 2016). Why is it the case is not clear, but it is likely that CCSs represent a favorable environment for signaling by clustering in small areas not only the receptors but also many scaffolding proteins and effectors. Some particular subsets of CCSs seem to represent an extreme versions of such signaling platforms. Indeed, it has long been observed that some cell types display particular clathrin-coated structures on the top of the canonical, endocytosis-competent CCSs. These large, long-lived and flat clathrin structures are termed clathrin-coated plaques (Figure 28). Although canonical CCSs can bud at the rim of plaques, the core of plaques themselves remains flat for hours and thus, do not support endocytosis. It has recently been demonstrated that the formation of these plaques relies on $\alpha\beta 5$ integrin (Baschieri et al. 2018; Zuidema et al. 2018). This integrin strongly anchors the clathrin machinery to the substrate, thus opposing budding forces. Signaling receptors such as the EGFR and c-Met can still accumulate at plaques and this seems to reinforce their signaling abilities. Although the precise mechanisms of this signaling reinforcement are not known, the very long-lived nature of plaques may play a role. In addition, it was shown that the formation of plaques depends on the rigidity of the substrate as these structures only assemble on rigid environments. Because their formation do not depend on the actin cytoskeleton, this makes plaques very peculiar mechanosensing structures that instruct the cell about substrate stiffness through regulating receptors signaling.

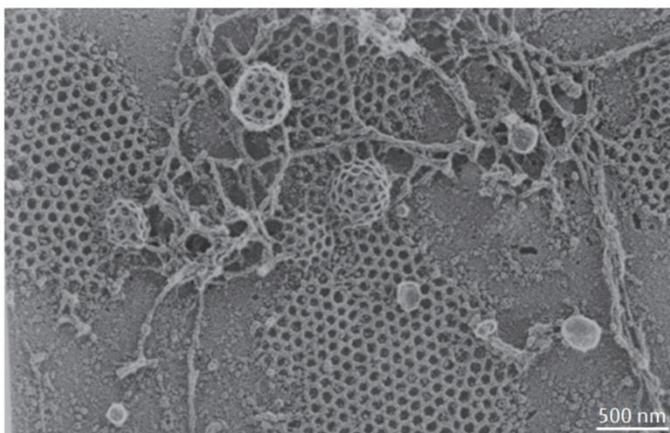


Figure 28. Clathrin structures can adopt two different shapes at the inner leaflet of the plasma membrane. They can either be invaginated clathrin-coated pits that perform endocytosis and which lattices are composed of both pentagons and hexagons. Clathrin can also adopt a flat hexagon-only structure which do not perform endocytosis. (From Humphries and Way, 2013)

II.2.2.2 - Role of CCSs in adhesion

Plaques have also been proposed to serve as adhesive structures (Maupin and Pollard 1983) and indeed, the recent discovery of their enrichment in $\alpha\text{v}\beta 5$ integrin supports this hypothesis (Baschieri et al. 2018). Independently of this latter report, $\alpha\text{v}\beta 5$ -rich reticular adhesions have been described and reported to play a critical role in cell adhesion during mitosis. These reticular adhesions are also independent of the actin cytoskeleton and proteomic analyzes revealed the presence of many CCSs components in these structures. Clathrin-coated plaques and reticular adhesions are probably actually the same structures (as discussed in Lock et al. 2019), highlighting the prominent role of at least some CCSs in cell adhesion. Although plaques have been proposed to slow down migration (Saffarian, Cocucci, and Kirchhausen 2009), presumably because of their strong adhesion to the substrate, observations from the lab show that many highly migratory cell types such as fibroblasts display such structures.

We also recently discovered a new type of CCSs that forms at contact sites between the plasma membrane and collagen fibers and that are called tubular clathrin/AP-2 lattices (TCALs) (Elkhatib et al. 2017; Figure 29). TCALs specifically nucleate along collagen fibers as a consequence of the deformation the fiber imposes to the plasma membrane. This creates a local membrane curvature that is a signal triggering nucleation. Indeed, many CCSs proteins show a preferential binding to curved membranes (Pucadyil and Holkar 2016). In addition, TCALs are longer-lived than non-fiber-engaged CCSs probably because they are rich in $\beta 1$ -integrin that bind the fiber. While canonical, budding CCSs display a spherical shape, TCALs adopt the morphology of the fiber to form a tubular clathrin structure that wrap around and pinch collagen fibers (Figures 29). We showed that, in addition to FAs, TCALs allow the cell to efficiently grab collagen fibers. This adhesive role of TCALs is completely independent of endocytosis as clathrin inhibition does not perturb their formation and function. This may seem contradictory but, in the absence of clathrin, the core of AP-2 and other adaptors can still assemble, whether on fibers or at any location of the plasma membrane, and can still recruit receptors (Elkhatib et al. 2017). However, in the absence of clathrin, budding and endocytosis are completely inhibited. In the 3D environment composed of collagen fibers, FAs are less prominent than in 2D and are only detected at the very leading edge and the very back of the cell. As a consequence, when the cell extends a protrusion, the tension quickly raises across the

protrusion. Because TCALs form anchoring points to the fibers all around the cell, they reduce these tension as measured in laser ablation experiments (Elkhatib et al. 2017). This allows the cell to develop long, stable protrusions and in fine, it allows the cell to migrate efficiently in the 3D environment.

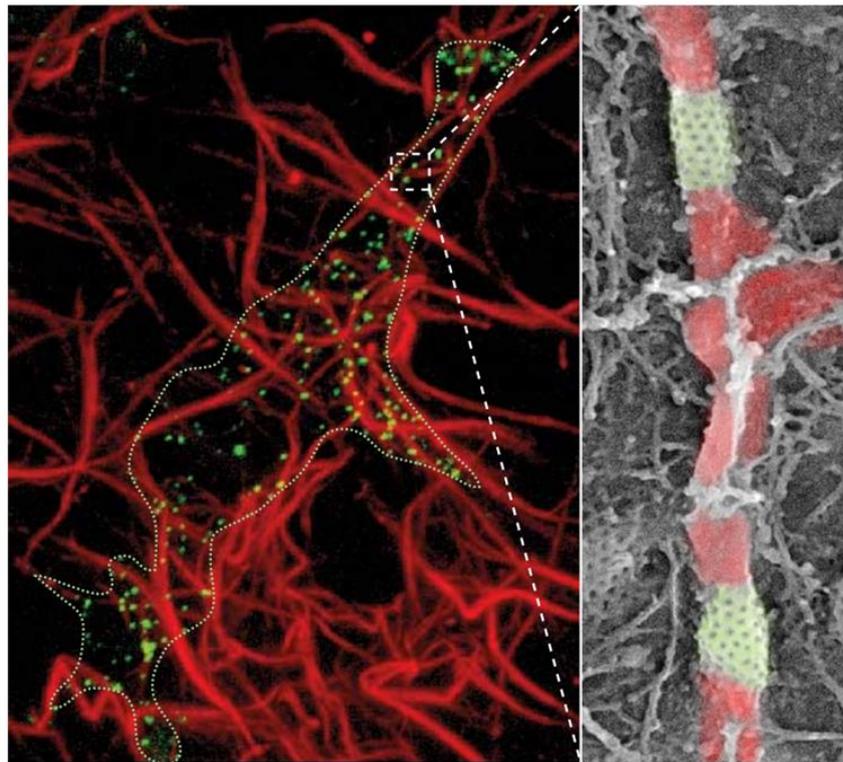


Figure 29. We have recently shown that CCSs can be used as adhesion structures. To do so they engage and accumulate along collagen fibers (left). They form tubular structures that pinch the fibers (right) and that were termed tubular clathrin/AP-2 lattices (TCALs) (From Elkhatib et al., 2017)

III - THE EPIDERMAL GROWTH FACTOR RECEPTOR

Growth factors are membrane anchored or secreted proteins that regulate many aspects of cellular functions. Some cell types secrete growth factors in the extracellular space. These growth factors then diffuse in the extracellular space until they bind and activate their receptor on the same cell (autocrine activation) or on other cells. This process allows is at the heart of cell-cell communication. Signaling induced by growth factor receptor affects most of cells function including cell proliferation, differentiation, migration, survival, metabolism tuning and cell-cycle control. Among the many described growth factors, the epidermal growth factor (EGF) is known to bind to its receptor (EGFR) that is a member of the receptor tyrosine kinase family (RTKs).

III.1 - EGF AND EGFRs FAMILY

58 RTKs have been identified in human, they are classified within 20 families. Among these families there is the family of EGFR, insulin receptor, PDGFR, VEGFR, FGFR or c-Met. If all RTKs families are different they still share some similarities in molecular architecture, activation mechanisms and induced signaling pathways.

III.1.1 - The receptor tyrosine kinase family

Regarding their molecular architecture, RTKs are transmembrane proteins that all share an extracellular ligand-binding domain and a tyrosine kinase domain (TKD) in their cytoplasmic tail. In the accepted scheme of RTKs activation, ligand-induced RTK dimerization leads to the transphosphorylation of the dimer through TKDs of individual monomers coming in close contact to the other monomer's cytoplasmic tail (Ullrich and Schlessinger 1990). Ligand-induced dimerization of RTK can happen through two main kind of mechanisms: the two ligands are either physically bound to form a bridge between the two receptors or the ligands induce conformational change that favors direct interaction of the monomers (Figure 30). Phosphorylated tyrosines in specific domains of receptor cytoplasmic domains represent docking sites for effectors containing phosphotyrosine-binding (PTB) or Src homology-2 (SH2)

domains. PTB and SH2 domains-containing scaffolding proteins further recruit other signaling complexes and effectors. This ultimately leads to signaling pathways activation.

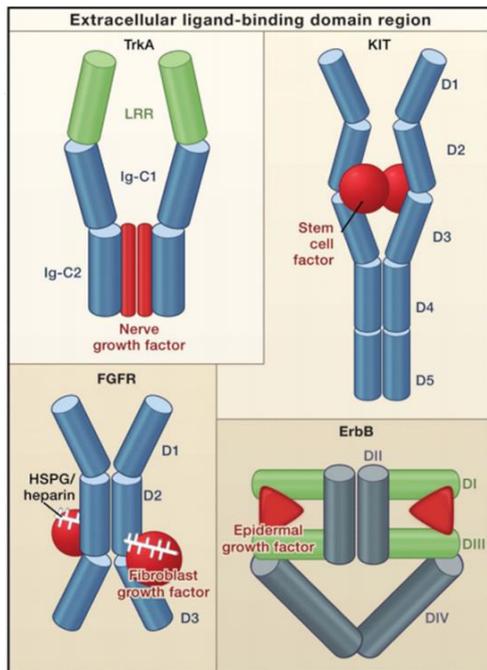


Figure 30. A) Ligand-induced dimerization of RTK can happen through two main kinds of mechanisms: the two ligands are either physically bound to form a bridge between the two receptors (e.g. the receptors TrkA and KIT) or the ligands induce conformational change that favors direct interaction of receptor monomers (e.g. the receptors FGFR and ErbB). (From Lemmon and Schlessinger, 2011)

Many of the signaling proteins are common to multiple RTKs but each RTK have a very precise cellular effect. This raises the question of how activation of the same effectors by different RTKs lead to different final consequences. RTKs are mostly internalized through CME (Goh and Sorokin 2013). Like all transmembrane proteins, RTKs are nonspecifically constitutively internalized at a very low rate. Because the constitutive recycling rate is higher than the constitutive endocytosis rate, RTKs are mostly localized at the plasma membrane at steady state. Ligand-binding induces RTKs internalization at a much higher rate and can lead to RTKs degradation in the endosomal system. These two mechanisms synergize to control the depletion of RTKs from the cell surface upon their acute stimulation, thus protecting the cell from overstimulation.

III.1.2 - The EGFR family

The EGFR family is one of the most studied RTK due to its early discovery and its major role in physiology and diseases, including cancers. They are ubiquitously expressed in both epithelial and mesenchymal cells.

III.1.2.1 - EGFR family members

The EGFR family is composed of four receptors named HER1-4 or ErbB1-4 that are encoded by genes located on different chromosomes. HER1 corresponds to the canonically termed EGFR. All members of the EGFR family are essential as their knockdown is lethal (Citri and Yarden 2006). ErbB2 is unable to bind ligands and ErbB3 does not possess a kinase activity. All members of the ErbB family are able to dimerize with the other members.

The structure of ErbB receptors are divided into four extracellular domains, the transmembrane domain and three intracellular domains. The extracellular domain I and III are enriched in leucine residues and are responsible for ligand binding. The extracellular domain II and IV are enriched in cysteine residues and are responsible for disulfide bond formation. The domain II is also responsible for the dimerization of the receptor. Their cytoplasmic tail is composed of a juxtamembrane domain, a tyrosine kinase domain (TKD) and finally the C-terminal tail (Figure 31).

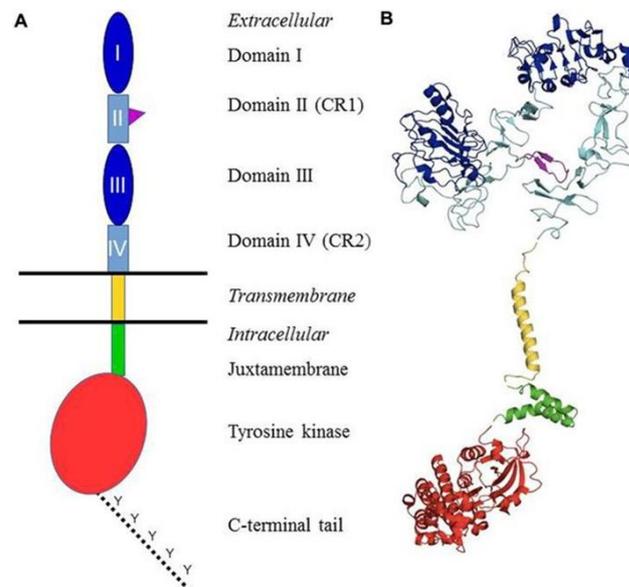


Figure 31. The structure of ErbB receptors are divided into four extracellular domains, the transmembrane domain and three intracellular domains. The extracellular domains I and III are enriched in leucine residues and are responsible for ligand binding. The extracellular domain II and IV are enriched in cysteine residues and are responsible for disulfide bond formation. The domain II is also responsible for the dimerization of the receptor. Their cytoplasmic tail is composed of a juxtamembrane domain, a tyrosine kinase domain (TKD) and finally the C-terminal tail (Appert-Collin et al., 2015)

III.1.2.2 - Ligands of the EGFR family

The EGFR family have eleven known ligands: EGF, heparin-binding epidermal growth factor-like growth factor (HB-EGF), betacellulin, TGF- α , epiregulin, amphiregulin, epigen and 4 different neuregulins. All these ligands bind specifically to the different members of the EGFR family. All ErbB dimers combinations, plus all ligands combinations, give a total of a 614 theoretically possible dimer/ligand complexes (Roskoski 2014). The different ligands lead to dimers with different stability and this finally leads to different signaling outcomes (Freed et al. 2017). All ligands are first exocytosed as transmembrane precursors at the surface of the secreting cell prior to be cleaved and thus released in the extracellular space.

III.1.2.3 - The epidermal growth factor

EGF is the prototypic ligand of the EGFR family. It was first identified in the submaxillary gland (Cohen 1960). EGF is a small peptide of 53 amino acids and, like all ErbB ligands, it is derived from a larger precursor, the pre-pro-EGF comprising 1207 amino acids. Pre-pro EGF contains a transmembrane domain and mature, soluble EGF is later released upon cleavage by a metalloprotease (Le Gall et al. 2003). Soluble EGF is folded via three disulfide bonds. EGF has a strong affinity for EGFR with a K_d of 1.77×10^{-7} M (Kuo et al. 2015). Within the ErbB family, EGF is only able to bind to EGFR.

EGF can be found in many body fluids including milk, saliva and plasma (Carpenter and Cohen 1979). EGF plasma level in mice is about 1 ng/mL (Byyny et al. 1974). EGF have positive effects on cell survival, proliferation and migration and (Hernandez et al. 2009) is notably involved in cancer cell invasion (Hernandez et al. 2009).

III.2 - PHYSIOLOGY OF THE EGFR

Because of its importance in physiology and diseases, the EGFR is one of the most studied receptors. It is also the target for different clinically relevant drugs. Although it is now studied for decades, many of its basics are still not completely understood, or are still a matter of debate.

III.2.1 - EGFR activation

For instance, EGFR activation process is a multistep, sequential process that is highly regulated and is the target of specific inhibitors.

III.2.1.1 - EGFR dimerization

ErbBs dimerization is based on receptor conformational change rather than on the ligands physically bridging the two monomers (Garrett et al. 2002). Ligand binding to the domains I and III induces a conformational change that disrupts interactions between domain II and IV. This leads to the unmasking of a dimerization domain in the domain II (Burgess et al. 2003). The exposure of the dimerization domain allows the interaction with a second ligand-bound receptor monomer (Figure 32). If it is mostly accepted that EGF is responsible for the dimerization of its receptors some studies proposed that it can also bind and activate preformed receptor EGFR dimers (Clayton et al. 2005; Gadella and Jovin 1995). Along that line, EGFR overexpression, or EGFR experimental crosslinking, is known to induce the autoactivation of the receptor through uncontrolled dimerization, in the absence of ligands (Endres et al. 2013; Lu et al. 2010)

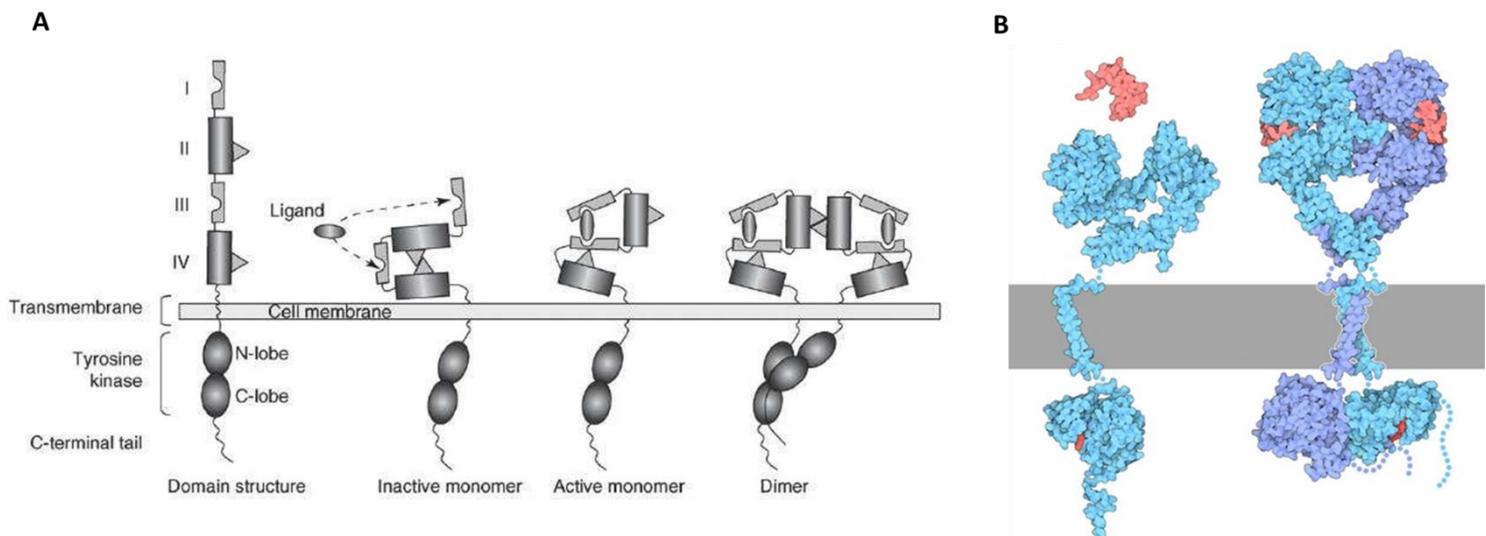


Figure 32. A) Ligand binding to domains I and III induces a conformational change that disrupts interactions between domain II and IV. This leads to the unmasking of a dimerization domain in the domain II. The exposure of the dimerization domain allows the interaction with a second ligand-bound receptor monomer. (From Sergina and Moasser, 2007) B) Epidermal growth factor (red) and its receptor (blue). The inactive form (left) dimerizes when it binds to the hormone (right). (<https://pdb101.rcsb.org/motm/126>)

III.2.1.2 - EGFR transphosphorylation

Activation of the tyrosine kinase domains of the ErbB family is different from the process at stake in other RTKs. Reaching the active conformation does not require the activation loop phosphorylation. Upon ligand binding, TKDs from the two EGFR monomers interact together. This interaction induces a conformational change in TKDs, allowing them to act as genuine tyrosine kinases (Zhang et al. 2006). Once activated, the TKDs control the transphosphorylation of different tyrosine residues on the cytosolic tail of each EGFR monomers. This initiates the activation of the signaling cascade through allowing the recruitment of specific effectors.

III.2.1.3 - EGFR inhibitors

Because EGFR signaling plays a major role in cancers and other diseases, many inhibitors have been developed that aim at inhibiting its activation. These drugs can be divided in two categories: monoclonal antibodies that target the extracellular domains of EGFR, and tyrosine kinase inhibitors (TKI). Monoclonal antibodies have been developed against ErbB1 and 2 and are used in clinics (eg. cetuximab and trastuzumab). They prevent ligand binding/dimerization, which leads to signaling inhibition. TKIs have been developed against all members of the ErbB family (eg. gefitinib and erlotinib). They act intracellularly by binding to the ATP-binding site of TKDs, thus preventing ATP binding and kinase activity. Unlike monoclonal antibodies, TKIs do not prevent ligand binding and dimerization (Lichtner et al. 2001).

III.2.2 - EGFR signaling

Activated EGFR displays multiple phosphorylated tyrosine on its cytoplasmic tail. These phosphorylated tyrosine residues, together with surrounding residues, form docking sites for adaptor proteins that control different downstream signaling pathways, some of which are briefly described below (Roskoski 2014; Figure 33).

Among these pathways, the extracellular regulate kinas (Erk) pathway of the mitogen activated protein kinase (MAPK) pathways is one of the most important. This specific MAPK pathway is triggered by the recruitment of Grb2 to activated EGFR through its SH2 domain. This leads to the activation of the guanine nucleotide exchange factor (GEF) Sos. Sos activates

Ras by catalyzing the exchange of GDP for a GTP on this GTPase. Ras then activates Raf (a serine/threonine protein kinase). Raf dimerizes and autophosphorylates before to phosphorylate/activate the Mek1/2 kinases which then phosphorylate/activates the Erk1/2 kinases. Upon activation, Erk1/2 translocates from the cytosol to the nucleus where it activates other kinases and transcription factors. Ultimately, the Erk/MAPK pathway regulates the transcription of many different genes whose expression level mostly regulate cell survival, proliferation and cell differentiation.

Another critical signaling pathway regulated by the EGFR is the PI3K/Akt pathway. PI3K is a heterodimer made of the association of a p85 regulatory subunit and a p110 (or PI3KCA) catalytic subunit. PI3K can be directly activated by EGFR-associated Grb2. PI3K phosphorylates PIP2 into PIP3. PIP3 then recruits the kinase Akt at the plasma membrane through its PIP3-binding PH domain. Akt then phosphorylates both mTOR and BAD. The phosphorylation events controlled by Akt activates mTOR but inhibits BAD. The PI3K/Akt pathways is involved in cell survival and apoptosis. By hydrolyzing PIP3 into PIP2 PTEN negatively regulate the PI3K pathway.

The PLC- γ /PKC pathway relies on the SH2 domain-dependent recruitment of PLC- γ to activated EGFR. PLC- γ hydrolyzes PIP2 into IP3 and DAG. These second messengers regulates many intracellular events, and in particular, they synergize to activate the protein kinase C (PKC). PKC phosphorylates many substrates including Raf. This pathway regulates cell proliferation, cell survival, migration and cell adhesion.

EGFR activation also leads to the activation of Signal Transducers and Activators of Transcription (STATs) through controlling their phosphorylation. This depends on the recruitment of the non-receptor kinase Src onto activated EGFR that then phosphorylates STATs. In addition, EGFR-regulated Erk1/2 can also phosphorylates STATs on different residues, reinforcing their activity. Once activated, STATs translocate to the nucleus to act as transcription factors regulating proliferation, differentiation, cell survival and apoptosis.

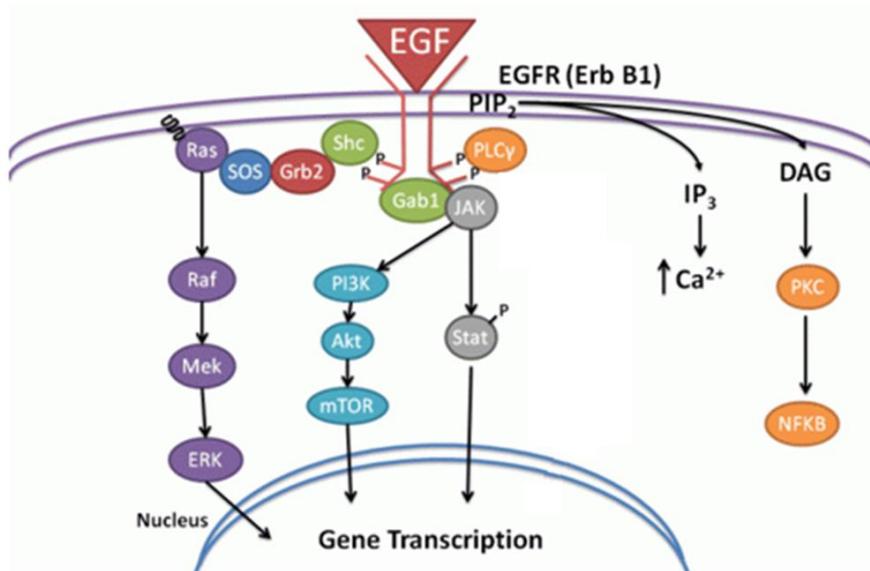


Figure 33. EGFR downstream signaling is composed of different pathways: the MAPK pathway (violet), the PI3K/Akt pathway (blue), the Jak/STAT pathway (grey) and the PLC/PKC pathway (orange). (From <https://pancreapedia.org>)

Finally, it is well documented that growth factors receptors (and in particular the EGFR) and integrin can synergize and even generate some cross-talk mechanism to initiate or reinforce signaling pathways activation (Moro et al. 1998; Moro et al. 2002; Schneller, Vuori, and Ruoslahti 1997; Sridhar and Miranti 2006). Indeed, integrin and growth factor receptors share many signaling effectors and FAs are home to intense signaling. Cross-talk interaction have been sorted into 4 categories (Ivaska and Heino 2011; Figure 34): 1) Concomitant signaling, in which integrins and EGFR signal independently to the same signaling pathways (Mettouchi et al. 2001), 2) collaborative signaling, in which activated integrins gather signaling effectors and RTK thus enhancing signalization (Goel et al. 2004), 3) direct activation, in which some integrins can directly activate EGFR without the binding of ligand (Moro et al. 1998) and finally 4) amplification of signaling, in which integrin activation increases the amount of EGFR at the plasma membrane (Moro et al. 2002).

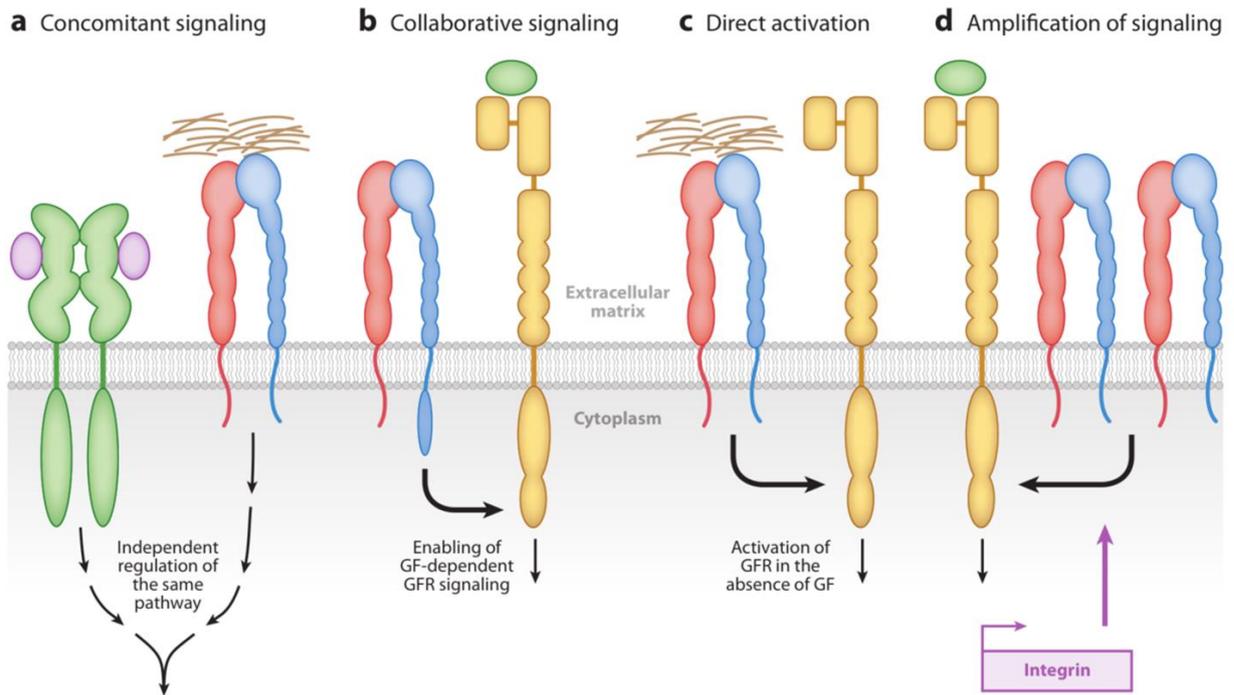


Figure 34. Crosstalk between RTKs and integrins can be sorted into four mechanisms. a) Concomitant signaling, in which integrins and RTKs signal independently to the same signaling pathways, b) collaborative signaling, in which activated integrins gather signaling effectors and RTKs thus enhancing signalization, c) direct activation, in which some integrins can directly activate RTKs without the binding of ligand and finally d) amplification of signaling, in which integrin activation increases the amount of RTKs at the plasma membrane. (From Ivaska and Heilo, 2011)

III.2.3 - EGFR endocytosis

EGFR endocytosis is a very complex, highly regulated process that involves different endocytosis motifs carried by its cytosolic tails, but also different adaptors and even different endocytosis pathways. Once internalized, the fate of the EGFR may also vary depending on the conditions of its stimulation.

III.2.3.1 - Mechanisms of EGFR uptake

The EGFR can be internalized through CME but also through some clathrin-independent pathways. At low EGF concentration (below 10ng/ml), the EGFR is internalized only via CME. At higher concentrations, the EGFR is also internalized through clathrin-

independent pathways. EGFR endocytosis in clathrin-independent pathways relies on the ubiquitination of its cytosolic tail (Sigismund et al. 2005). CME of EGFR is associated with sustained signaling and recycling to the plasma membrane. On the opposite, when EGFR is endocytosed through clathrin-independent pathways, it is more likely to reach lysosomes and to be degraded (Sigismund et al. 2008). The mechanisms supporting this clathrin-independent pathway are still elusive but may involve a recently proposed, very fast pathway relying on endophilins (Boucrot et al. 2015). However, EGFR internalization through clathrin-independent pathways is still a matter of debate (Madhus and Stang 2009) and the observed differences could be cell type specific.

There is a larger consensus that EGFR uptake is supported by CME. Yet, precise mechanisms of EGFR recruitment into CCSs are also debated. Some studies reported that EGFR activation (autophosphorylation) is required for its efficient recruitment at CCSs (Lamaze and Schmid 1995; Sorkina et al. 2002). Other studies, however, proposed that EGFR dimerization is sufficient to trigger its endocytosis and can thus happen without activation of the kinase domain of the receptor (Wang, Chen, and Wang 2015; Wang, Villeneuve, and Wang 2005). These studies proposed that the dimerization allows the formation of a pair of dileucine (LL)-based endocytic motifs that constitute the basic unit for binding to AP-2. Finally, there are also evidences that experimentally-induced EGFR clustering is sufficient to trigger its endocytosis (Heukers et al. 2013).

More precisely, some mechanisms have been proposed to regulate EGFR recruitment at CCSs. First, a direct interaction have been reported between EGFR and AP-2 (Sorkin et al. 1996). Also, activated EGFR recruit the ubiquitin ligase Cbl that ubiquitinates EGFR itself as well as some EGFR effectors. Ubiquitynated EGFR is recognized by the clathrin accessory proteins Eps15 and epsin that participate in the recruitment of EGFR into CCSs (Kazazic et al. 2009). Grb2 as well as lysine residues acetylation are also involved in addressing the EGFR to CCSs (Goh et al. 2010). It is also worth saying that the intracellular domain of EGFR is the only among the ErbB family to promote active endocytosis (Baulida et al. 1996). In any case, it is clear that EGFR is targeted to CCSs through different, redundant mechanisms that cooperate to ensure that this receptor does not stay at the plasma membrane once activated.

Some studies have suggested that, upon EGF-binding, the EGFR is able to trigger a local CCSs nucleation in order to promote its own endocytosis (Johannessen et al. 2006). On the opposite, some other investigators found that activated EGFR is targeted to pre-existing CCSs

(Rappoport and Simon 2009). Similarly intriguing, the absolute requirement of AP-2 for EGFR internalization is also debated (Motley et al. 2003; Pascolutti et al. 2019). It appears that the experimental conditions, like EGF incubation at 4°C before performing endocytosis assays, critically affect EGFR physiology (Benmerah and Lamaze 2007). In physiological conditions, AP-2 is required for EGFR endocytosis (Huang et al. 2004) and EGFR is targeted to pre-existing CCSs. However, these different findings highlight the complexity of EGFR endocytosis and its sensitivity to environmental factors.

III.2.3.2 - EGFR intracellular trafficking

2 to 5 minutes after its endocytosis, EGFR-containing-vesicles fuse with early endosomes. The pH in early endosome is not low enough to promote EGF unbinding from the receptor and thus the receptor continues to signal from endosomes. The early endosomes then mature to progressively become late endosomes and multivesicular bodies. EGFR is observed in perinuclear MVBs 15-20 min after its endocytosis (Carpentier et al. 1987; Figure 35). As EGF does not separate from its receptor, the entire EGF/EGFR complex is recycled to the plasma membrane. As mentioned above, a portion of internalized EGFR can recycle back to the plasma membrane, especially if the receptor was internalized through CME. Recycling can occur through a fast pathway, from early endosomes, or through a slow pathway, from late endosomes and specialized Rab11-positive recycling endosomes (Sorkin et al. 1991). Most of the internalized EGFR, however, is not recycled to the plasma membrane but rather routed to lysosomes for degradation. EGFR ubiquitynation is essential for lysosomal targeting (Huang et al. 2006). Of note, the different possible ligands of EGFR lead to different endocytic fates. TGF- α associates weakly to EGFR as compared to EGF and thus dissociates from EGFR in the acidic environment of early endosomes, leading to more EGFR being recycled (Longva et al. 2002).

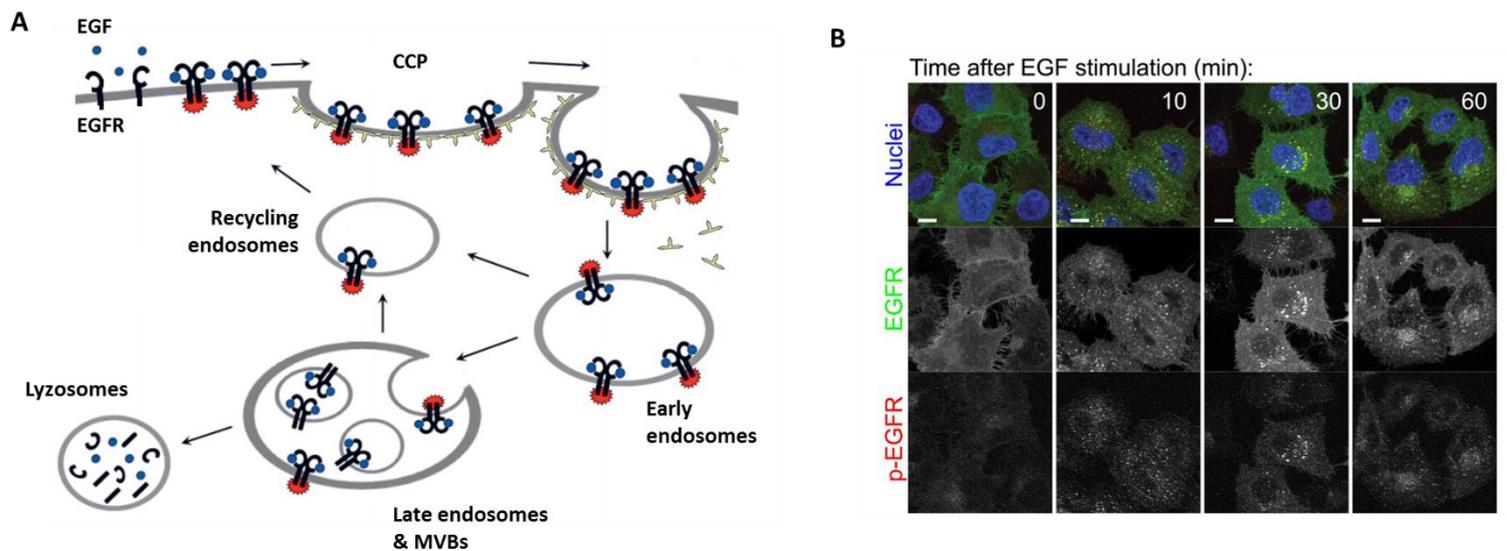


Figure 35. EGF endocytosis. A) EGF binding to its receptor leads to their recruitment into CCPs. The EGF/EGFR complex traffic into endosomes until they are either degraded or recycled back to the plasma membrane. (Adapted from Göstring, 2011) B) Immunofluorescence images of the progressive EGFR activation and internalization. Scale bars, 10 μ m. (From Villaseñor et al., 2015).

III.3 - EGFR IN CELL MIGRATION

EGFR regulates several aspects of cell migrations. First, EGFR directly modulates cell motility by regulating both cell adhesion to its substrate and the cytoskeleton that powers cell movement. In addition, gradients of EGFR ligands can lead to directed cell migration. Finally, EGFR also regulates the organization of the ECM supporting cell migration.

III.3.1 - EGFR in cell motility

EGFR activation is known to enhance cell motility. Both TGF- α and EGF are able to promote migration in different cell lines (Morelli et al. 1992; Westermark, Magnusson, and Heldin 1982). EGFR overexpression in tumor cells leads to enhanced motility and invasion (Xue et al. 2006). EGF is also known to promote cell migration by reactivating the epithelial to mesenchymal transition (Grände et al. 2002). EGFR regulates directly cell migration by fine tuning the activity of two major actors of the motility machinery: adhesions to the substrate and the actin cytoskeleton.

EGFR first modulates cell adhesion by regulating the composition of FAs (Eberwein et al. 2015). EGFR also regulates FAs turnover by promoting their disassembly (Xie et al. 1998) through downregulating FAK (Z. Lu et al. 2001). EGFR activation can also regulate adhesion by regulating the expression level of integrins, their expression at the cell surface as well as their own signaling activity at the plasma membrane (Laura Moro et al. 2002). Finally, EGFR can increase cell spreading in cells adhering to rigid matrices (Saxena et al. 2017).

EGFR signaling also affects migration by regulating the actin cytoskeleton. EGF stimulation modulates actin rearrangement (Rijken et al. 1991) and promotes lamellipodia extension (Segall et al. 1996). EGF-induced actin rearrangement relies on cofilin, which severs actin filaments. EGF stimulation leads to PLC activation which, in turn, activates cofilin. This leads to barbed ends availability and thus more sustained actin polymerization (Mouneimne et al. 2004)

III.3.2 - EGFR in chemotaxis

EGFR also regulates directed cell migration both *in vitro* and *in vivo*. *In vitro*, EGFR has been shown to regulate the persistence of migration in one given direction in 2D (Maryse Bailly et al. 2000) and in 3D (Raja et al. 2010). Cells are also able to migrate towards an immobilized gradient of EGF in 3D (Fisher et al. 2018). Both EGFR expression level and EGF gradient shape modulate the cell ability to migrate towards EGF (Fisher et al. 2018; S.-J. Wang et al. 2004)

In vitro, when exposed to an EGF gradient, cells protrude and show F-actin enrichment towards the gradient (M. Bailly et al. 1998). To migrate up the gradient, the cell needs to polarize its actin machinery. This is possible because EGF/EGFR-mediated regulation of actin polymerization remains localized (Kempiak et al. 2003). While EGFR localization is homogeneous at the plasma membrane, its endocytosis is asymmetrically regulated (Bailly et al. 1998) and thus could explain the local effect of EGF. In fact, cofilin regulation by EGF remains local via LIM kinase that spatially restricts activated cofilin at the leading edge. This induces local actin polymerization and further directed migration (Yamaguchi and Condeelis 2007; Figure 36)

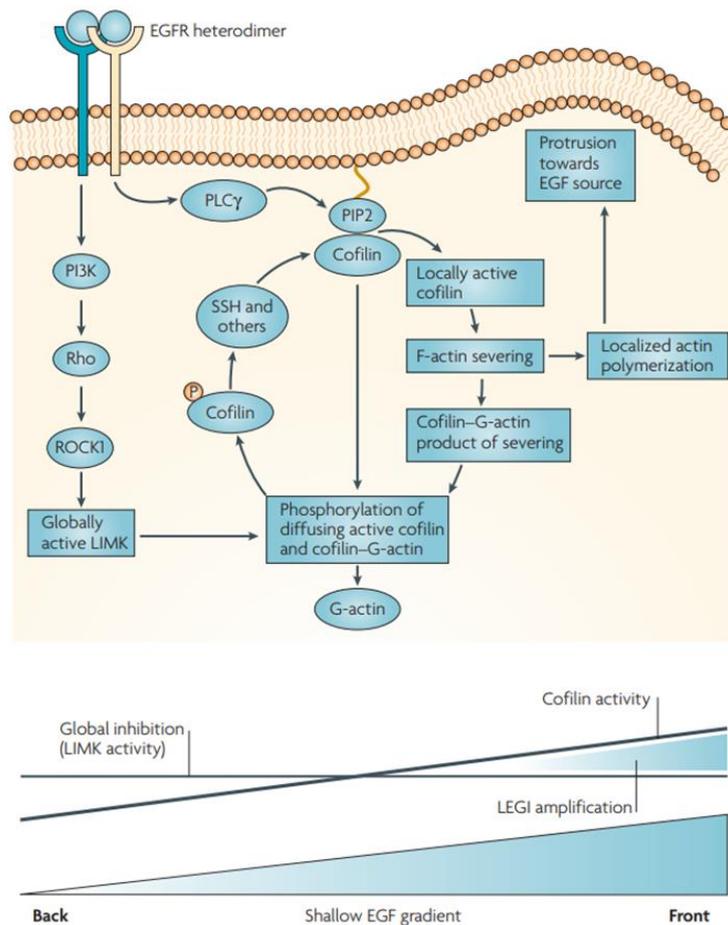


Figure 36. A model for EGF-driven chemotaxis. In the cell, cofilin is globally inactivated by the LIMK activity. At the front of the cell, higher concentration of EGF induces a higher activation of cofilin that locally overcomes the LIMK activity. Thus actin polymerization only pushes the membrane at the leading edge, maintaining directed migration towards the EGF gradient. (From Wang et al., 2007)

In vivo, the directed migration of cancer cells toward an EGF source has been observed by intravital imaging (Wyckoff et al. 2004); Figure 37). A paracrine loop involving EGF and CSF-1 is proposed to drive this cancer cell invasion. EGF secreted by macrophages attract tumors cells which in turn secrete CSF-1 that recruit macrophage. This leads to a positive recruitment loop that promotes directed invasion (Wyckoff et al. 2004); Figure 37).

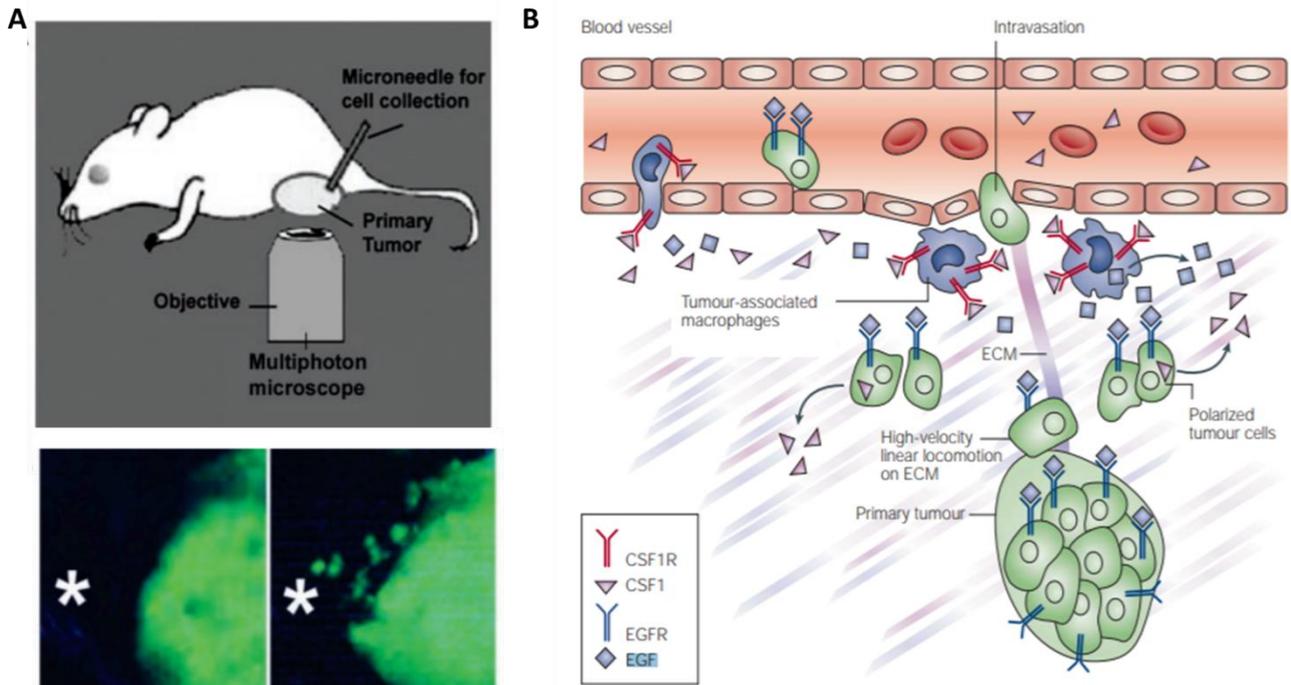


Figure 37. A) Cells leaving a tumor to migrate towards an EGF gradient diffusing from a needle (*) (Wyckoff et al., 2004). B) A paracrine loop involving EGF and CSF-1 is proposed to drive this cancer cell invasion. EGF secreted by macrophages attract tumors cells which in turn secrete CSF-1 that recruits macrophage. This leads to a positive recruitment loop that promotes directed invasion (Condeelis and Segall, 2003)

III.3.3 - EGFR and the ECM

EGFR also indirectly influences cell migration through regulation of the ECM. EGFR can both trigger ECM protein secretion and remodeling of the existing matrix. Conversely, matrix properties also influences EGFR signaling.

EGF stimulation leads to increased expression and secretion of collagen (Laato et al. 1987) and fibronectin (Mimura et al. 2004). EGFR can also participate to matrix remodeling, notably through regulating the expression of matrix metalloproteases. EGFR activation leads to increased expression of collagenase (Pilcher et al. 1999) and the expression of MMPs is impaired in EGFR deficient cell (Kajanne et al. 2007). Nevertheless, EGF can increase or decrease MMPs expression depending on cell types and experimental conditions (Bouchard et al. 2010; S. Kim et al. 2009). The increase in MMPs expression take part in the larger program of EGF-induced EMT (Wilkins-Port and Higgins 2007). Finally, the EGF ability to induce cell

contraction and thus matrix remodeling is still debated (Kim et al. 2015; Yang, Lin, and Yu 1997).

If EGFR signaling affects the ECM, the presence of some ECM components also increases EGFR signaling (Cybulsky, McTavish, and Cyr 1994). Changes in the matrix composition leads to modifications of the EGF-induced gene expression pattern (Yarwood and Woodgett 2001). Finally, the substrate rigidity is well known to affect EGFR signaling via integrin/EGFR crosstalk signaling (Saxena et al. 2017) and this play an important role in regulating cell migration.

AIM OF THE THESIS

Cell migration occurs during the whole life of every individual as a part of embryonic development and maintenance of physiological functions but it also plays a role in several pathologies. It is powered by the actin cytoskeleton that provides the force required to push forward the plasma membrane of the leading edge. This force leads to an actual movement of the cell only if the actin cytoskeleton is physically engaged with the extracellular matrix via adhesion structures. To date, different types of adhesion complexes have been described, that, for most of them, display a similar composition and depend on connections with the actin cytoskeleton. In addition we recently proposed that CCSs could also serve as adhesion structures.

CCSs are primarily known as the structures supporting clathrin-mediated endocytosis (CME). During CME, cells internalize membrane portions and their associated receptors or cargoes. Because of the large range of receptors it internalizes, CME is key to many cellular processes. It starts with the recruitment of receptors and their ligands inside membrane invagination that are called clathrin-coated pits. Some of these cargoes have been proposed to initiate CCSs formation and modulate their dynamics. These pits progressively invaginate until scission from the plasma membrane and formation of a vesicle that further traffic inside the cell.

We showed that a subset of CCSs, called tubular Clathrin/AP-2 lattices (TCALs), can also serve as adhesion structures to collagen fibers. Cell adhesion and the cell's capacity to grab collagen fibers were inhibited by disruption of TCALs. We first observed that CCSs accumulated along collagen fibers in the 3D environments. TCALs nucleation is triggered by membrane curvature induced by collagen fibers contacting the plasma membrane. TCALs further adopt the tubular morphology of collagen fibers, wrapping around and pinching the fiber. This morphology is rather different from the circular shape adopted by canonical CCSs that regulate cargoes internalization.

From these, we hypothesized that matrix-bound CCS ligands could link the two roles of CCSs: their well-documented role as endocytic structures and their newly discovered role as adhesion structures. These matrix-bound ligands could modulate TCAL dynamics and thus

affect 3D cell migration. In extension, gradient of matrix-bound CCS ligands could lead to directed migration.

The main objective of my PhD was thus to establish the link between the endocytic and adhesive role of CCSs during cell migration. This main question can be divided in several specific objectives:

- Obtain amenable collagen fibers coated with CCSs ligand, as 3D networks are difficult to manipulate in order to design specific experiments.
- Investigate the consequences of ligand-coated collagen fibers on TCALs and their dynamic.
- Investigate the forces applied to ligand-coated collagen fibers and the potential role of TCALs.
- Investigate a potential effect of collagen-bound ligands on 3D, directed migration and the potential role of TCALs in this process.

I started by developing a protocol to obtain individual collagen fibers coated with EGF or LDL. I then found that collagen-bound ligands increase the local nucleation of CCSs, leading to more TCALs being associated with EGF/LDL-coated collagen fibers. I later found that this increased in TCALs recruitment induces an increase in the forces applied by the cell to fibers. I finally observed that in 3D, cells migrate toward EGF-coated collagen fibers using a mechanism that relies on TCALs. In conclusion, I propose a model of directed migration driven by TCALs in response to substrate-bound CCS-ligands. In this model, an asymmetrical distribution of ligands induces an asymmetrical nucleation and thus distribution of adhesive clathrin structures. This leads to an asymmetric distribution of forces applied to the ECM, ultimately leading to directed migration.

RESULTS

Title:

Clathrin-coated structures support 3D haptotaxis through local force transmission

Authors:

Enzo Bresteau¹, Francesco Baschieri¹, Nadia Elkhatab¹ and Guillaume Montagnac¹

Affiliations:

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

Corresponding authors: enzo.bresteau@gustaveroussy.fr and

guillaume.montagnac@gustaveroussy.fr

Abstract

Migrating cells navigates in complex environments through sensing and interpreting biochemical and/or mechanical cues. Gradients of cytokines or growth factors orient cell migration through a complex integration of local, intracellular signaling events. Similar mechanisms are believed to govern cell migration during haptotaxis, when cells follow signals emanating from substrate-anchored chemoattractants. Here, we report that recently identified tubular clathrin/AP-2 lattices (TCALs), a subset of clathrin-coated structures (CCSs) that pinch collagen fibers, mechanically control haptotaxis along fibers decorated with ligands of CCS cargoes, in 3D environments. We observed that epidermal growth factor (EGF) or low density lipoprotein (LDL) bound to collagen fibers lead to an increased local nucleation and accumulation of TCALs. By using engineered, mixed collagen networks, we demonstrate that this mechanism selectively increases local forces applied on ligand-decorated fibers. We show that these effects depend on ligand's receptors but do not rely on their ability to trigger signaling events. The accumulation of TCALs along ligand-decorated fibers steers migration in 3D environments. We conclude that ligand-regulated, local TCAL nucleation results in asymmetric force distribution that orient cell migration in 3D environments.

Main Text

INTRODUCTION

Clathrin-mediated endocytosis is a fundamental process that regulates the uptake of a wide diversity of cell-surface receptors and their ligands. By doing so, clathrin-coated structures (CCSs) impinge on many cellular functions including cytokinesis (Montagnac, Echard, and Chavrier 2008), cell migration, and cell invasion (Maritzen, Schachtner, and Legler 2015). We recently demonstrated that in 3D environments composed of collagen fibers, β 1-integrin-enriched CCSs wrap around and pinch the fibers, thus offering many anchoring points that facilitate cell migration (Elkhatib et al. 2017). These collagen fiber-pinching CCSs, also called tubular clathrin/AP-2 lattices (TCALs), are frustrated in nature as they try and fail to internalize fibers that are longer than the cell itself. Yet, although their lifetime is longer as compared to non fiber-engaged CCSs, it is still limited in time suggesting that they may be able to bud and to produce endocytic vesicles after an initial period of frustration on fibers. Collagen fibers are sticky structures to which many proteins can bind or adsorb. Besides other extracellular matrix (ECM) components, some cytokines and growth factors can also directly or indirectly bind to collagen fibers (Hynes 2009; Schuppan et al. 1998). For example, the epidermal growth factor (EGF) was reported to bind to collagen fibers with an estimated affinity of 1.706 μ M (Yang et al. 2009). The EGF receptor (EGFR) is mostly internalized through CCSs and this has been suggested to play a role in chemotaxis towards EGF gradients (Wyckoff et al., 2004; S.-J. Wang et al. 2004). Low density lipoprotein (LDL), the ligand of another major CCS cargo, was also reported to bind to collagen fibers (Nivelstein-Post et al. 1994). We set out here to investigate the relationship between TCALs and two major CCSs cargos, and their role in orienting cell migration in 3D environments.

RESULTS

Production and characterization of ligand-decorated collagen fibers

We observed that incubating Alexa488-labelled EGF with a pre-polymerized fibrillar collagen gel leads to an accumulation of EGF along fibers (Supplementary Fig. 1a), thus confirming previous findings (Yang et al. 2009). Because such a gel is difficult to handle in order to address specific questions, we setup a protocol to produce, decorate with EGF, and manipulate individual collagen fibers (Fig. 1a). Our protocol produces collagen fibers of quite homogenous length that can be spotted on glass or incorporated into a 3D network (Fig. 1b and c). These fibers were homogeneously decorated with Alexa488-EGF (Fig. 1b and c) and similar results were obtained when using Dil-LDL (Supplementary Fig. 1b). Fluorescence recovery after photobleaching (FRAP) assays showed that the fluorescence associated with Alexa488-EGF does not recover when engineered fibers were spotted on 2D surfaces or incorporated into 3D networks (Supplementary Fig. 1c and d). If free EGF was added in the medium before the FRAP experiments, a mobile fraction of approximately 50% was detected (Supplementary Fig. 1c and d). The immobile fraction observed in these latter experiments most likely correspond to the EGF fraction that is stably associated with collagen fibers recovered at the end of our production protocol.

We observed that fiber-associated EGF was able to activate the mitogen activated protein kinase Erk as efficiently as free EGF alone, or free EGF added together with non-decorated fibers, when MDA-MB-231 cells and fibers were incubated together in suspension (Supplementary Fig. 2a and b). Akt could not be activated by free EGF alone in these conditions, but was equally activated by EGF-decorated fibers and by free EGF added together with non-decorated fibers (Supplementary Fig. 2a and c). These latter results probably reflect the requirement of integrins engagement for efficient activation of the Akt pathway, this is conflict with previously reported experiment (Velling, Stefansson, and Johansson 2008). In

addition, MDA-MB-231 cells were able to internalize fiber-bound EGF as evidenced by the transfer of EGF-associated fluorescence from the fibers to internal compartments (Supplementary Fig. 2d). Together, these data demonstrate that collagen fiber-associated EGF is functional and can be sensed and internalized by cells.

Exacerbated accumulation of TCALs along ligand-decorated fibers

We next took advantage of our protocol to generate composite 2D substrates by sequentially spotting EGF-decorated and non-decorated fibers on glass coverslips (Fig. 1d). When MDA-MB-231 cells were seeded for 15 min on this mixed, 2D network, we noticed that CCSs marked with the α -adaptin subunit of the clathrin adaptor AP-2 accumulated along non-decorated fibers as previously described (Elkhatib et al. 2017) (Fig. 1d). However, CCSs accumulated approximately 30 % more along EGF-decorated fibers as compared to naked fibers, whether using Alexa-488-labelled EGF or unlabeled EGF (Fig. 1d and e). We also observed that the EGFR accumulated along EGF-decorated fibers but not along non-decorated fibers (Fig. 1f and g image and quantif). The accumulation of TCALs along EGF-decorated fibers was dependent on the expression of the EGFR but was insensitive to Gefitinib, a drug that inhibits the kinase activity of EGFR (Cohen et al. 2004) (Fig. 1e). We controlled that Gefetinib was indeed able to inhibit EGFR-triggered Erk activation (Supplementary Fig. 3a and b). These results suggest that EGFR activation (autophosphorylation) and downstream signaling pathways are not playing a role in the accumulation of TCALs along EGF-decorated fibers. We next performed live cell imaging of genome-edited MDA-MB-231 cells engineered to express GFP-tagged, endogenous μ 2-adaptin subunit of AP-2. When these cells were allowed to spread for 15 min on the composite, 2D network, we measured that TCALs average lifetime was slightly increased on EGF-decorated as compared to non-decorated fibers, although the difference was not statistically significant (Fig 2a-c). However, TCALs had approximately 20%

more chances to nucleate on EGF-decorated fibers as compared to control fibers (Fig. 2b and d). EGFR knockdown, but not Gefetinib treatment, abolished the preferential TCALs nucleation rate on EGF-decorated fibers (Fig. 2e). It has been suggested that, in particular experimental setups, EGF/EGFR complexes could induce the de novo formation of CCSs (Wilde et al. 1999; Johannessen et al. 2006). In addition, receptors are known to take an important part in the maturation of nascent CCSs (Ehrlich et al. 2004; Loerke et al. 2009) and experimental clustering of cell-surface receptors can induce the de novo formation of CCSs (Liu et al. 2010). Thus, it is possible that local accumulation of EGFR driven by collagen fiber-associated EGF results in local TCAL nucleation/maturation as suggested by our data. The role of EGFR kinase activity in EGFR interaction with CCSs has been supported by several studies (Lamaze and Schmid 1995; Sorkina et al. 2002) but some others suggested that EGF-induced receptor dimerization or clustering is sufficient to be recruited at CCSs (Wang, Villeneuve, and Wang 2005; Wang, Chen, and Wang 2015; Heukers et al. 2013). In any case, our results suggest that EGF/EGFR-regulated local TCAL nucleation does not require EGFR activation. Along this line, we observed that EGF still accumulates at CCSs in cells seeded on glass and treated with Gefetinib, although less efficiently than in control cells (Supplementary Fig. 3c and d). It is possible that the particular conditions of TCALs assembly, which is driven by a cooperation between local membrane curvature and β 1-integrin engagement (Elkhatib et al. 2017), are further favored by high local concentrations of functional CCS cargoes. In agreement with that, we observed that TCALs also preferentially accumulated along Dil-LDL-decorated fibers as compared to non-decorated ones (Fig. 2f and g). Similar to EGF-decorated fibers, CCS nucleation rate was increased on LDL-decorated fibers as compared to naked fibers (Fig. 2h). Thus, local accumulation of CCS ligands drives the local accumulation of TCALs through increased CCS nucleation.

Local TCALs accumulation regulates local forces applied on collagen fibers

We noticed that cells spreading on the mixed 2D network developed more protrusions along EGF-decorated fibers as compared to non-decorated fibers (Fig. 3a and b). Yet, we did not observe any differential enrichment of $\beta 1$ -integrin on one or the other type of fiber (Supplementary Fig. 4a and b). Similarly, the focal adhesion marker vinculin was equally distributed between EGF-decorated and non-decorated fibers (Supplementary Fig. 4c and d) suggesting that these adhesion structures did not participate in the differential protrusion activity on the two different types of fibers. We previously reported that TCALs help cells to develop long protrusions in 3D collagen networks by providing several anchoring points to collagen fibers (Elkhatib et al. 2017). Here, our data suggest that the increased accumulation of TCALs along EGF-decorated fibers allow the cell to develop longer protrusions on these fibers as compared to non-decorated fibers. To check whether cells specifically exert more forces on ligand-decorated fibers as compared to normal fibers, we developed a collagen fiber remodeling assay. EGF-decorated and non-decorated fibers were sequentially spotted on a glass coverslip and MDA-MB-231 cells were allowed to remodel this composite network for 2 hours. Collagen fiber remodeling was characterized by a transition from rod-like shape to circular aggregates as cells pulled on and packed fibers on their dorsal surface. In the course of this process, TCALs were observed colocalizing and moving together with collagen fibers on the dorsal surface of cells (Supplementary Fig. 5a and b). In order to precisely quantify fiber remodeling, we measured the evolution of fibers circularity over time. The data show that EGF-decorated fibers are almost twice as much remodeled as compared to non-decorated fibers over a 120 min period (Fig. 3c-e). The preferential remodeling of EGF-decorated fibers was dependent on EGFR expression (Fig. 3f and Supplementary Fig. S5c). However, Gefetinib treatment did not modulate the preferential remodeling of EGF-decorated fibers indicating that EGFR activation is not required (Fig. 3f and Supplementary Fig. S5c). Knockdown of AP-2 subunits, but not

clathrin heavy chain (CHC), abrogated the preferential remodeling of collagen fibers (Fig. 3f and Supplementary Fig. S5c). This is consistent with our previous characterization of TCALs showing that clathrin is not required for the accumulation of AP-2-positive structures along collagen fibers and that it does not play a role in the adhesive function of TCALs (Elkhatib et al. 2017). Of note, inhibiting the formation of focal adhesions by using talin-specific siRNAs strongly reduced the global fiber remodeling rates but did not inhibit the preferential remodeling of EGF-decorated fibers (Fig. 3f and Supplementary Fig. S5c). Together, our data show that both EGFR and TCALs are required for the preferential remodeling of EGF-decorated collagen fibers. In addition, we observed that LDL-decorated fibers were also preferentially remodeled over non-decorated fibers and that this also depended on AP-2 (Fig. 3g and h). Together, our data suggest that the accumulation of TCALs along CCS ligands-decorated fibers leads to more forces being applied on them.

Local TCALs accumulation orients cell migration in 3D

We previously reported that TCALs help cells to migrate in 3D environments by serving as adhesive structures to collagen fibers (Elkhatib et al. 2017). We hypothesized that the preferential accumulation of TCALs along ligands-decorated fibers could further favor cell migration in 3D networks. We first observed that MDA-MB-231 cells located in 3D network composed of EGF-decorated fibers were more elongated as compared to cells located in a non-decorated network (Fig. 4a-c). Together with our previous results showing that TCALs help cells to build long protrusions required for migration (Elkhatib et al. 2017), these data suggested that EGF on collagen fibers further potentiates cell elongation through favoring TCALs formation. However, cells migrated with a similar velocity in the EGF-decorated and non-decorated networks (Supplementary Fig. 5d). It is possible that a homogenous distribution of EGF-decorated fibers around the cell leads to a global stabilization of all cell protrusions,

without a net consequence on cell displacement in one given direction. Stable protrusions pointing in different directions could hinder a potential effect of EGF-decorated fibers on cell migration. To test this possibility, we aimed at observing cells located in an asymmetric network in which EGF-decorated fibers would be restricted to a defined area of the gel. For this, EGF-decorated, or non-decorated, chopped collagen fibers produced as in Fig. 1a were added to a mixture of non-polymerized collagen in a 1:1 ratio. A first mix containing the non-decorated fibers was deposited as a 50 μ l drop on a glass coverslip at 4°C. 6 μ l of the second mix, containing the EGF-decorated fibers, was carefully injected into the first one and the composite gel was then allowed to polymerize. This protocol enabled the production of a composite network with a clear segregation between EGF-decorated and non-decorated fibers, without creating a physical interface between the two regions (Fig. 4d). We observed that cells located in the homogenous EGF-decorated or non-decorated areas of the gel migrated randomly in all directions (Fig. 4e). However, cells located at the boundaries between the two areas had approximately twice as much chances to migrate towards the EGF-decorated network rather than towards the non-decorated network (Fig. 4e and f). This preferential migration towards EGF-decorated fibers was dependent on EGFR and CCSs (Fig. 4f). Together, our results show that TCALs accumulation on CCSs ligands-decorated fibers allow cells to migrate towards this type of fibers.

Overall, we have found that TCALs strongly accumulate on CCSs ligands-decorated fibers because ligand/receptor complexes favor the local nucleation of TCALs on these fibers. The preferential accumulation of TCALs on ligands-decorated fibers allows the cell to exert more forces on these fibers as compared to non-decorated fibers. As a consequence, cells preferentially migrate towards ligands-decorated fibers in 3D environments. Strikingly, this haptotactic mode of migration does not depend on the transduction of signaling pathways but

only on the local accumulation of TCALs that allow cells to pull harder on collagen fibers decorated with CCSs ligands. Thus, it is important to consider that haptotaxis is not a simple variation of chemotaxis but is actually driven by completely different mechanisms, at least in the case of EGF and LDL in 3D collagen networks. Because ECM fibers are abundant in complex organisms, and many chemoattractants and other potential CCSs ligands are known to bind to ECM, the mechanism we described here may play a central role in cell migration in different contexts, from development to cancers.

METHODS

Cell lines and constructs

MDA-MB-231 cells (a gift from P. Chavrier, Institut Curie, Paris, France) or genome edited MDA-MB-231 cells engineered to express an endogenous GFP-tagged or mCherry-tagged $\mu 2$ subunit (a gift from D. Drubin, University of California-Berkeley, California, USA) were grown in DMEM Glutamax (Gibco) supplemented with 10% foetal calf serum (Gibco) at 37°C in 5% CO₂.

Antibodies, growth factors and drugs

Rabbit polyclonal anti- α -adaplin antibodies (M300) were purchased from Santa Cruz Biotechnology Inc. Mouse monoclonal anti- α -adaplin antibodies (ab2807) were purchased from Abcam. Activated integrin (4B4) antibody (6603113) was obtained from Beckman coulter. Alexa488-conjugated anti-mouse (A21202) or anti-rabbit (A21202) antibodies and Alexa545-labelled phalloidin (A22283) were from Molecular Probes. Anti phospho-Akt (9271), Erk1/2 (9102) and phospho-Erk1/2 (9101) antibodies were purchased from Cell Signaling. HRP-conjugated anti-mouse antibodies (115-035-062) for western blot and Cy3-conjugated anti-rabbit (711-165-152) or anti-mouse (711-165-152) antibodies were purchased from Jackson ImmunoResearch Laboratories. HRP-conjugated anti-rabbit antibodies (A0545) for western blot were purchased from Sigma.

Gefitinib (CDS022106) was purchased from Sigma and used at a final concentration of 10 μ M unless otherwise stated. Before experiment, cells with gefitinib were pre-treated for 30 min at 37°C. Alexa Fluor® 488 (E-13345)- or 647 (E35351)-labelled EGF and DiI-conjugated LDL (L3482) were purchased from Thermo Fisher. Rat tail Collagen-I (CA10483-01) was purchased from GIBCO. Human recombinant EGF (E9644) was purchased from Sigma.

RNA interference

For siRNA depletion, MDA-MB-231 cells were plated at 50% confluence and treated with the indicated siRNA (30 nM) using RNAimax (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Cells were used after 72 h or 120 h of siRNA treatment as shown by immunoblotting analysis with specific antibodies. Equal loading of the cell lysates was verified by immunoblotting with anti-tubulin antibodies.

The following siRNAs were used: Talin, 5'-ACAAGAUGGAUGAAUCAUUUUU-3'; μ 2-adaptin, 5'-AAGUGGAUGCCUUUCGGGUCA-3'; Clathrin heavy chain (CHC), 5'GCUGGGAAAACUCUUCAGATT-3'; α -adaptin, 5'-AUGGCGGUGGUGUCGGCUCTT-3'; Epidermal growth factor receptor (EGFR) 5'-GAGGAAAUAUGUACUACGA-3' (EGFR-1) and 5'-GCAAAGUGUGUAACGGAAUAGGUAU-3' (EGFR-2); non-targeting siRNAs (siControl), ON-TARGETplus Non-Targeting SMARTpool siRNAs (Dharmacon D-001810-01).

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 using Pierce[™] Coomassie Plus (Bradford) Assay Kit (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. Analyzes of bands densitometry were performed using ImageJ.

For testing Gefitinib-mediated inhibition of EGFR signaling, 200 000 MDA-MB-231 were serum-starved for 2h, and then stimulated or not with 10 ng/ml EGF in the presence or not 10

μ M gefitinib for 5 minutes at 37°C. Cells were then harvested, lysed and analyzed by western blot as describe above.

EGF/LDL-decorated collagen fibers and 3D networks

200 μ l of a mix containing a 10:1 ratio of unlabeled collagen type I and Alexa548(or Alexa488 or Alexa647)-labelled collagen type I (Gibco, A1048301) at a final concentration of 1.1 mg/ml was allowed to polymerize in a 1.5 ml Protein LoBind Tube (Eppendorf, 0030108116) for 12 min at room temperature. 200 μ l of PBS were then added on ice to the mix before 3 rounds of 10s sonication were performed using a Q125 sonicator at 40% amplitude (Qsonica sonication). 2.5 μ l of EGF/LDL was then added (or not) to the mix before incubation at room temperature for 2.5h (final concentration EGF: 1.25 μ g/ml and LDL: 6.25 mg/ml). 600 μ l of PBS was then added to the mix before 12 rounds of 10s sonication were performed at 40% amplitude. Polymerized, sonicated, EGF-or LDL- or non-decorated collagen fibers were then pelleted by centrifugation for 1h at 45000 rpm (centrifuge 5427R; rotor FA-45-30-11; Eppendorf) at 4°C. The pellet was washed twice with cold PBS before to be either resuspended in 1 ml cold PBS (for coverslip spotting experiments) or incorporated into another mix for 3D collagen network preparation. Fiber solution was kept on ice to prevent collagen fibers aggregation.

For collagen fibers deposition on glass (2D), 100 μ l of the mix containing polymerized, sonicated collagen fibers were spotted for 10 min on a 12 mm coverslip (Marienflied, 0111520) or in a glass-bottom 96 well plate (Greiner, 07-000-630) at room temperature before to be washed twice with PBS. For sequential deposition experiments, naked fibers were spotted as described, then washed twice with PBS before EGF- or LDL-decorated fibers were spotted for 10 min as well and washed twice using PBS.

For incorporation into 3D networks, the pellet composed of polymerized, sonicated collagen fibers was resuspended in a 200 μ l mix containing non-polymerized collagen and 45 μ l of this

new mix were deposited in a glass-bottom 96 well plates pretreated with poly-L-lysine 0.1% (Sigma, 8920) for 10 min. For some experiments, the mix contained 200 cells/ μl . The mix was allowed to polymerize at room temperature for 30 min before to be covered with complete medium. For generating composite 3D networks, 7 μl of a mix containing EGF- or LDL-decorated, polymerized, sonicated fibers and 200 cells/ μl was gently pipetted inside a 45 μl mix containing naked, polymerized, sonicated fibers and 200 cells/ μl that was deposited on glass a few seconds before. All steps were performed at 4°C to prevent polymerization. The composite 3D network was then allowed to polymerize at room temperature for 30 min before to be covered with complete medium.

Indirect immunofluorescence microscopy and fluorescence quantification

MDA-MB-231 cells plated for 15 min on the top of naked and EGF- or LDL-decorated fibers spotted on coverslips were fixed in ice-cold methanol or PFA and processed for immunofluorescence microscopy by using the indicated antibodies. Cells were imaged through a 100 \times 1.40NA UPlanSApo objective lens of a wide-field IX73 microscope (Olympus) equipped with an Orca-Flash2.8 CMOS camera (Hamamatsu) and steered by CellSens Dimension software (Olympus).

For anti-vinculin staining, cells were briefly extracted for 1 min using 0.1% Triton prior to fixation.

For calculating the degree of CCSs or integrins or vinculin alignment along collagen fibers, naked and decorated fibers were segmented using ImageJ software and the average fluorescence intensity of the anti- α -adapin or anti-integrin or anti-vinculin staining in fibers area was measured for both type of fibers and for each individual cells and normalized to the area occupied by respective collagen fibers. For protrusions quantification, the proportion of

protrusions associated with each type of fibers were manually counted in ImageJ. At least 50 cells per conditions were quantified in 3-5 independent experiments.

Total internal reflection fluorescence microscopy

For total internal reflection fluorescent microscopy (TIRF), MDA-MB-231 cells seeded on glass or onto collagen-fibers-coated glass coverslips were imaged through a 100x 1.49 NA TIRF objective lens on a Nikon TE2000 (Nikon France SAS, Champigny sur Marne, France) inverted microscope equipped with a QuantEM EMCCD camera (Roper Scientific SAS, Evry, France / Photometrics, AZ, USA), a dual output laser launch, which included 491 and 561 nm 50 mW DPSS lasers (Roper Scientific), 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.

To measure EGFR accumulation on collagen fibers, MDA-MB-231 cells were transfected with a EGFR-GFP encoding plasmid (Addgene #32751) using linear PEI (MW 25.000, Polysciences) and following manufacturer's instructions. Cells were allowed to spread for approximately 15 min on the top of a mixed network composed of naked and EGF-decorated fibers spotted onto a glass-bottom fluorodish (World Precision Instruments, FD35-100) before to be imaged for 5 min. EGFR-associated average fluorescence intensity was quantified in ImageJ using the above described segmentation protocol at time points showing the greatest accumulation of EGFR on fibers. At least 20 cells per condition were analyzed in 2 independent experiments.

To monitor EGF recruitment at CCSs, genome-edited MDA-MB-231 cells on fluorodishes were starved for 2 h and treated or not with 10 mM Gefitinib for 1h before to be incubated in the presence of the same concentration of Gefitinib and 50 ng/ml Alexa488-EGF before to be imaged for 10 min. For quantification, CCSs were individually segmented using ImageJ and average EGF-associated fluorescence was measured at the time point showing the maximum

colocalization between EGF and CCSs. At least 2000 CCSs from at least 5 cells per condition and per experiments were quantified in 2 independent experiments.

Fluorescence Recovery After Photobleaching (FRAP)

EGF-decorated collagen fibers were spotted on glass or embedded in a 3D gel as described above in the presence or not of 8 $\mu\text{g/ml}$ Alexa488-EGF. FRAP was performed on a Leica Sp8 confocal microscope equipped with a Pecon incubation chamber to maintain the cells at 37°C and 5% CO₂ and using the FRAP wizard of the Leica software. One fiber was manually selected and subjected to 100% laser power. One frame was collected before photo-bleaching, and 40 frames were collected after bleaching to analyze fluorescent recovery at the frequency of 1 frame/30 sec. Data were analyzed using the ImageJ FRAP Profiler plugin (McMaster University, Canada) to extract recovery curves and calculate the half-time recovery.

Fiber-associated EGF stimulation and internalization assays in suspension

For fiber-associated EGF stimulation assays, MDA-MB-231 cells were starved for 2h before to be harvested using trypsin and incubated in suspension alone or in the presence of 50 ng/ml Alexa-488 EGF, or naked fibers, or Alexa488-EGF-decorated fibers, or naked fibers and 50 ng/ml Alexa488-EGF as indicated. The amount of fibers used in the assay was chosen so that the concentration of Alexa488-EGF on EGF-decorated fibers was equivalent to 50 ng/ml soluble EGF as determined by SDS-PAGE followed by EGF-associated fluorescence quantification using a gel imager (Biorad ChemiDoc XRS+ System) and ImageJ software. Cells were incubated at 37°C for 5 or 30 minutes before cells were harvested at 4°C and subjected to lysis in cold MAPK buffer followed by western-blot analysis using the indicated antibodies. For fiber-associated EGF internalization assays, the same protocol was used except that fibers were labelled using Alexa548-conjugated collagen and cells were incubated only with

Alexa488-EGF-decorated fibers at 37°C for 5, 10, 20 or 40 minutes before to be harvested and fixed using PFA. Fixed cells were centrifuged for 2 min at 500g and resuspended in 500 μ L PBS. Cells were then spotted on a coverslip and imaged by epifluorescence microscopy.

Spinning disk microscopy

Control or siRNA-treated, genome edited MDA-MB-231 cells were imaged for exposure times of 200 ms 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 100 mW DPSS lasers (Andor). Images were acquired with a Zyla sCMOS camera (Andor). The system was steered by IQ3 software (Andor).

For calculating CCSs lifetime on collagen fibers, naked or EGF- or LDL-decorated collagen fibers were segmented using ImageJ software and CCSs were tracked using the trackmate plugin. Tracks corresponding to CCSs detected on only one frame were discarded. For calculating the CCSs nucleation index, each new appearance of a μ 2-adaptin-mCherry marked CCSs were manually counted on segmented fibers and results were expressed as a function of fiber length and time. At least 700 CCSs from at least 15 cells per condition and per experiments were analyzed in 3-5 independent experiments.

Collagen fibers remodeling assay

Naked and EGF- or LDL-decorated fibers were sequentially spotted on glass-bottom 96-well plates before 40 000 control or siRNA-treated or Gefitinib-treated MDA-MB-231 cells were seeded per well in DMEM supplemented with 1% FCS. Plates were then immediately imaged at 37°C and 5% CO₂ by spinning disk microscopy. One frame was collected every 20 minutes for 6 hours. Naked and decorated fibers were individually segmented and remodeling over time

was quantified as a function of the evolution of collagen fibers circularity index using the following ImageJ macro:

```
for (i = 0; i < 10; i++) {  
run("Subtract Background...", "rolling=10 stack");  
setAutoThreshold("Default dark");  
//run("Threshold...");  
setOption("BlackBackground", false);  
setThreshold(XX,100000);  
run("Convert to Mask", "method=Default background=Dark");  
run("Remove Outliers...", "radius=0.5 threshold=50 which=Dark stack");  
run("Analyze Particles...", "size=10-Infinity show=Nothing summarize stack");  
close();  
}
```

The threshold (XX) was manually defined for each fiber type and for each experiment. At least 6 wells per condition and per experiments were analyzed in 3-4 independent experiments.

3D migration assays

For spreading analysis in 3D, MDA-MB-231 were embedded in uniform 3D networks composed of either naked or EGF-coated collagen fibers in 96-well plates and imaged with a wide field microscope 24h later. Cell circularity was measured using ImageJ. At least 70 cells per condition and per experiments were quantified in 3 independent experiments. Data are expressed as ranked, inversed circularity.

For migration assays in composite 3D networks, control or siRNAs-treated MDA-MB-231 cells in the presence of DMEM supplemented with 2% FCS were imaged 24h after being embedded in the gel by spinning disk microscopy through a 10x objective by focusing on areas of the gel where both non-decorated and decorated regions were visible. Frames were collected every 20

min for 10 hours. Cells were manually tracked using Image J and separated into 3 categories: cells only evolving in the non-decorated network, cells only evolving in the decorated network, and cells reaching at some point the interface between decorated and non-decorated networks. In that latter case, initial tracking point was set when cells reached the interface, if they were not already at the interface at the beginning of the movie. At least 20 cells per condition and per experiments were quantified in at least 3 independent experiments. Data are represented via rose plot produced by the ImageJ plugin chemotaxis tool (Ibidi).

Statistical analyses

Statistical analyses in Fig. 1e, Fig. 2e, Fig. 3efgh, Fig. 4f and Fig. S5c have been performed using Kruskal-Wallis One Way Analysis of Variance (ANOVA) followed by an All Pairwise Multiple Comparison Procedure (Tukey Test). Data in Fig. 1g, Fig. 2cdgh, Fig. 3b, Fig. 4c, Fig. S3d, Fig. S4bd and Fig. S5d have been tested using Student's t-test. All statistical analyses were performed using SigmaStat software.

References

- Cohen, Martin H. et al. 2004. "United States Food and Drug Administration Drug Approval Summary: Gefitinib (ZD1839; Iressa) Tablets." *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 10(4): 1212–18.
- Ehrlich, Marcelo et al. 2004. "Endocytosis by Random Initiation and Stabilization of Clathrin-Coated Pits." *Cell* 118(5): 591–605.
- Elkhatib, Nadia et al. 2017. "Tubular Clathrin/AP-2 Lattices Pinch Collagen Fibers to Support 3D Cell Migration." *Science (New York, N.Y.)* 356(6343).
- Heukers, Raimond et al. 2013. "Endocytosis of EGFR Requires Its Kinase Activity and N-Terminal Transmembrane Dimerization Motif." *Journal of Cell Science* 126(Pt 21): 4900–4912.
- Hynes, Richard O. 2009. "Extracellular Matrix: Not Just Pretty Fibrils." *Science (New York, N.Y.)* 326(5957): 1216–19.
- Johannessen, Lene E. et al. 2006. "Activation of the Epidermal Growth Factor (EGF) Receptor Induces Formation of EGF Receptor- and Grb2-Containing Clathrin-Coated Pits." *Molecular and Cellular Biology* 26(2): 389–401.
- Lamaze, C., and S. L. Schmid. 1995. "Recruitment of Epidermal Growth Factor Receptors into Coated Pits Requires Their Activated Tyrosine Kinase." *The Journal of Cell Biology* 129(1): 47–54.
- Liu, Allen P., François Aguet, Gaudenz Danuser, and Sandra L. Schmid. 2010. "Local Clustering of Transferrin Receptors Promotes Clathrin-Coated Pit Initiation." *The Journal of Cell Biology* 191(7): 1381–93.
- Loerke, Dinah et al. 2009. "Cargo and Dynamin Regulate Clathrin-Coated Pit Maturation." *PLOS Biology* 7(3): e1000057.
- Maritzen, Tanja, Hannah Schachtner, and Daniel F. Legler. 2015. "On the Move: Endocytic Trafficking in Cell Migration." *Cellular and molecular life sciences: CMLS* 72(11): 2119–34.
- Montagnac Guillaume, Arnaud Echard, and Philippe Chavrier. 2008. "Endocytic Traffic in Animal Cell Cytokinesis." *Current Opinion in Cell Biology* 20(4): 454–61.
- Nivelstein-Post, P., G. Mottino, A. Fogelman, and J. Frank. 1994. "An Ultrastructural Study of Lipoprotein Accumulation in Cardiac Valves of the Rabbit." *Arteriosclerosis and Thrombosis: A Journal of Vascular Biology* 14(7): 1151–61.
- Schuppan, D. et al. 1998. "Collagens in the Liver Extracellular Matrix Bind Hepatocyte Growth Factor." *Gastroenterology* 114(1): 139–52.
- Sorkina, Tatiana, Fangtian Huang, Laura Beguinot, and Alexander Sorkin. 2002. "Effect of Tyrosine Kinase Inhibitors on Clathrin-Coated Pit Recruitment and Internalization of

- Epidermal Growth Factor Receptor." *The Journal of Biological Chemistry* 277(30): 27433–41.
- Velling, Teet, Anne Stefansson, and Staffan Johansson. 2008. "EGFR and B1 Integrins Utilize Different Signaling Pathways to Activate Akt." *Experimental Cell Research* 314(2): 309–16.
- Wang, Qian, Xinmei Chen, and Zhixiang Wang. 2015. "Dimerization Drives EGFR Endocytosis through Two Sets of Compatible Endocytic Codes." *Journal of Cell Science* 128(5): 935–50.
- Wang, Qian, Greg Villeneuve, and Zhixiang Wang. 2005. "Control of Epidermal Growth Factor Receptor Endocytosis by Receptor Dimerization, Rather than Receptor Kinase Activation." *EMBO reports* 6(10): 942–48.
- Wang, Shur-Jen et al. 2004. "Differential Effects of EGF Gradient Profiles on MDA-MB-231 Breast Cancer Cell Chemotaxis." *Experimental Cell Research* 300(1): 180–89.
- Wilde, A. et al. 1999. "EGF Receptor Signaling Stimulates SRC Kinase Phosphorylation of Clathrin, Influencing Clathrin Redistribution and EGF Uptake." *Cell* 96(5): 677–87.
- Yang, Yifan et al. 2009. "Collagen-Binding Human Epidermal Growth Factor Promotes Cellularization of Collagen Scaffolds." *Tissue Engineering. Part A* 15(11): 3589–96.

Acknowledgment

The authors wish to thank the imaging facilities of Gustave Roussy and Institut Curie for help with image acquisition. Core funding for this work was provided by the Gustave Roussy Institute and the Inserm and additional support was provided by grants from ATIP/Avenir Program, la Fondation ARC pour la Recherche sur le cancer, Le Groupement des Entreprises Françaises dans la Lutte contre le Cancer (GEFLUC) and from Institut Nationale du Cancer (INCA 2018-1-PL BIO-02-IGR-1) and the Fondation pour la Recherche Médicale (FRM DEQ20180339205) to GM. This project was supported by grant "Taxe d'apprentissage Gustave Roussy - 2016 - EB"

E.B designed and performed experiments, analysed results and wrote the manuscript. F.B and 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 guillaume.montagnac@gustaveroussy.fr or to enzo.bresteau@gustaveroussy.fr

Figure legends

Figure 1. **TCALs preferentially accumulate along ligands-decorated fibers.** **a**, Scheme representing the different steps of collagen fibers production and decoration with ligands. **b**, Alexa555-labelled (left panel), Alexa488-EGF (right panel)-decorated fibers produced as in a were spotted on a glass coverslip. Scale bar: 5 μm . **c**, Alexa488-EGF-decorated fibers (right panel) produced as in a were embedded in a 3D collagen network. Scale bar: 20 μm . **d**, Alexa488-labelled, EGF-decorated fibers (green) and Alexa555-labelled, non-decorated fibers (Naked, blue) were sequentially spotted on a glass coverslip and MDA-MB-231 cells were allowed to spread on this composite network for 15 min before to be fixed and stained for α -adaptin (red). Scale bar: 10 μm . **e**, Quantification of the enrichment of average α -adaptin staining fluorescence intensity on Alexa488EGF-, or native EGF-decorated fibers (as indicated) as compared to non-decorated fibers in MDA-MB-231 cells as in d and treated with the indicated siRNA or with Gefitinib. Data are expressed as the mean \pm SD percentage over average fluorescence on non-decorated fibers (* $P < 0.01$, One Way Analysis of Variance – ANOVA. $N=3$). **f**, MDA-MB-231 cells expressing GFP-tagged EGFR were allowed to spread on a composite network as in d. Higher magnification of boxed area are shown. Scale bar: 10 μm . **f**, Quantification of the enrichment of average GFP-EGFR fluorescence intensity on Alexa488EGF-decorated fibers as compared to non-decorated fibers in MDA-MB-231 cells as in d and treated or not with Gefitinib, as indicated. Data are expressed as the mean \pm SD ratio of EGF-fibers- versus naked fibers-associated GFP fluorescence.

Figure 2. **Increased nucleation rate of TCALs on EGF-decorated fibers.** **a**, Alexa488-labelled, EGF-decorated fibers (green) and Alexa555-labelled, non-decorate fibers (Naked, blue) were sequentially spotted on a glass coverslip and genome-edited MDA-MB-231 cells

expressing mCherry-tagged μ 2-adaptin (AP-2, red) were allowed to spread on this composite network for 15 min. Scale bar: 10 μ m. **b**, Kymographs of regions boxed in **a** over a 2 min time period. **c**, Quantification of the average lifetime of CCSs located on EGF-decorated or on non-decorated fibers as indicated (ns: non-significant, Student's t-test. N=3). **d**, Quantification of the average nucleation rate of CCSs located on EGF-decorated or on non-decorated fibers as indicated (* P<0.01, Student's t-test. N=3). **e**, Quantification of the ratio of the average nucleation rate of CCSs located on EGF-decorated versus non-decorated fibers in MDA-MB-231 cells treated with the indicated siRNA or with Gefitinib (* P<0.001, One Way Analysis of Variance – ANOVA. N=3). **f**, Alexa555-labelled, DiI-LDL-decorated fibers (green) and Alexa488-labelled, non-decorated fibers (Naked, blue) were sequentially spotted on a glass coverslip and MDA-MB-231 cells were allowed to spread on this composite network for 15 min before to be fixed and stained for α -adaptin (red). Scale bar: 10 μ m. **g**, Quantification of the average α -adaptin staining fluorescence intensity on DiI-LDL-decorated and non-decorated (Naked) fibers (* P<0.01, Student's t-test. N=3). Values for non-decorated fibers were set to 100%. **h**, Quantification of the average nucleation rate of CCSs located on DiI-LDL-decorated or on non-decorated fibers as indicated (* P<0.01, Student's t-test. N=3). All results are expressed as mean \pm SD.

Figure 3. TCALs allow cells to preferentially remodel ligands-decorated fibers. **a**, Alexa488-labelled, EGF-decorated fibers (green) and Alexa555-labelled, non-decorate fibers (Naked, blue) were sequentially spotted on a glass coverslip and MDA-MB-231 cells were allowed to spread on this composite network for 20 min before to be fixed and stained with phalloidin (red). Scale bar: 10 μ m. **b**, Quantification of the average percentage of non-decorated (Naked) or EGF-decorated fibers associated with protrusions in cells as in **a** (*

$P < 0.01$, Student's *t*-test. $N=3$). **c**, Alexa488-labelled, EGF-decorated fibers (green) and Alexa555-labelled, non-decorate fibers (Naked, red) were sequentially spotted on a glass coverslip. Scale bar: 100 μm . **d**, Kymographs depicting collagen fibers remodelling in the boxed area shown in **c** upon seeding MDA-MB-231 cells on the composite network for 120 min. **e**, Quantification of the average evolution of EGF-decorated and non-decorated (Naked) collagen fibers circularity upon cell seeding as in **d** (* $P < 0.01$, Student's *t*-test. $N=3$). **f**, Quantification of the ratio of EGF-decorated fibers versus non-decorated fibers circularity at $t=120$ min as depicted in **e** when using cells transfected with the indicated siRNAs or treated with Gefitinib (* $P < 0.001$, One Way Analysis of Variance – ANOVA. $N=3$). **g**, Quantification of the average evolution of LDL-decorated and non-decorated (Naked) collagen fibers circularity upon cell seeding (* $P < 0.01$, Student's *t*-test. $N=3$). **h**, Quantification of the ratio of LDL-decorated fibers versus non-decorated fibers circularity at $t=120$ min as depicted in **g** when using cells transfected with the indicated siRNAs (* $P < 0.001$, One Way Analysis of Variance – ANOVA. $N=3$). All results are expressed as mean \pm SD.

Figure 4. TCALs regulate 3D haptotaxis towards EGF-decorated fibers. **a**, MDA-MB-231 cells were embedded in a non-decorated fibers- (upper panel) or in EGF-decorated fibers (lower panel)-containing 3D collagen network and imaged 24h latter (representative images of three independent experiments). Scale bar: 10 μm . **b**, Quantification of cell elongation index corresponding to the inverse circularity index in 458 cells embedded in naked fibers (red)- or EGF fibers (green)-containing 3D networks from three independent experiments. Cells are sorted along the *x* axis from the most (left) to the less (right) elongated (1 on *y* axis=perfectly circular cell). **c**, Quantification of the mean \pm SD elongation index of cells embedded in naked fibers- or in EGF fibers-containing 3D networks, as indicated (* $P < 0.01$, Student's *t*-test. $N=3$).

d, Engineered composite 3D network comprising a non-decorated fibers (Naked, red)-containing area and an EGF-decorated fibers (green)-containing area in a supporting collagen gel was imaged by spinning disk microscopy. A phase contrast image of the same region of the composite network is shown. Scale bar: 20 μm . **e**, Rose plots representing the angular distribution of migration of MDA-MB-231 cells located in the EGF-fibers-containing area (top, green), the non-decorated fibers-containing area (bottom, red) or at the interface between the two areas (middle). **f**, Box plots representing the average ratio of cells initially located at the interface between the two areas as depicted in **d** and migrating towards the EGF-fibers-containing area versus the non-decorated fibers area SD (* $P < 0.001$, One Way Analysis of Variance – ANOVA). A ratio of 1 indicates no preferential migration towards one or the other area.

Supplementary figure legends

Supplementary Figure 1. **Characterization of ligands association with collagen networks.** **a**, Pre-polymerized, 3D collagen network was incubated for 30 min with 100 nM Alexa488-labelled EGF before to be imaged by spinning disk microscopy. Collagen fibers (left panel) were stained using an anti-collagen I antibody. Scale bar: 10 μm . **b**, Alexa555-labelled (left panel), Dil-LDL (right panel)-decorated fibers produced as in Fig. 1a were spotted on a glass coverslip. Scale bar: 5 μm . **c**, **d**, FRAP experiments of Alexa488-EGF on collagen fibers produced as in Fig. 1a and spotted on a glass coverslip (**a**) or embedded in a 3D network (**b**) with or without adding 100nM Alexa488-EGF (free EGF) in the medium 30 min before the beginning of the experiment, as indicated.

Supplementary Figure 2. **EGF on collagen fibers is functional.** **a**, MDA-MB-231 cells were serum-starved for 2h before to be trypsinized and incubated in suspension at 37°C with Alexa488-EGF-decorated or non-decorated fibers produced as in Fig. 1a and supplemented or not with soluble 30nM Alexa488-EGF for 5 or 30 min, as indicated. Cells were then harvested at 4°C and subjected to Western blot analysis using the indicated antibodies. Actin was used as a loading control. Control cells were incubated in suspension at 37°C for 5 min in the absence of fibers or EGF. **b**, **c**, Densitometry analyses of bands depicted in **a** when using the anti-phospho-Erk antibody (**b**) or the anti-phospho-Akt antibody (**c**). Controls were set to 1. **d**, MDA-MB-231 cells were serum-starved for 2h before to be trypsinized and incubated in suspension at 37°C with Alexa488-EGF-decorated fibers produced as in Fig. 1a for the indicated time periods. Cells were then fixed and stained with DAPI before to be imaged by spinning disk microscopy. Note that the initially green fibers (EGF-decorated) progressively

become only red (Alexa555-labelled collagen) as EGF is being internalized by cells. Scale bar: 5 μ m.

Supplementary Figure 3. **Gefitinib treatment does not prevent EGFR recruitment at CCSs.**

a, MDA-MB-231 cells were serum-starved for 2h before to be stimulated or not with 10nM EGF with or without 10 μ M Gefitinib for 5 min. Cells were then harvested and subjected to Western-blot analyses using the indicated antibodies. Total Erk (t-Erk) antibodies were used as a loading control. **b**, Densitometry analyses of bands depicted in **a** when using the anti-phospho-Erk antibody. Control was set to 1. **c**, Genome-edited MDA-MB-231 cells expressing mCherry-tagged μ 2-adaptin were serum-starved for 2h before to be stimulated with 30nM Alexa488-EGF for 5min in the presence or not of 10 μ M Gefitinib, as indicated, and imaged by TIRF microscopy. Scale bar: 2 μ m. **d**, Quantification of the average enrichment of Alexa488-EGF fluorescence intensity in CCSs over background (non-CCSs areas of the plasma membrane; * P<0.01, Student's t-test. N=3). Values for background were set to 0. All results are expressed as mean \pm SD.

Supplementary Figure 4. **β 1-integrin and vinculin equally distribute between EGF-decorate**

and non-decorated fibers. **a**, Alexa488-labelled, EGF-decorated fibers (green) and Alexa555-labelled, non-decorated fibers (Naked, blue) were sequentially spotted on a glass coverslip and MDA-MB-231 cells were allowed to spread on this composite network for 15 min before to be fixed and stained for β 1-integrin (red). Scale bar: 10 μ m. **b**, Quantification of the average β 1-integrin staining fluorescence intensity on EGF-decorated and non-decorated (Naked) fibers (ns: non-significant, Student's t-test. N=3). Values for non-decorated fibers were set to 100%. **c**, Alexa488-labelled, EGF-decorated fibers (green) and Alexa555-labelled, non-decorated

fibers (Naked, blue) were sequentially spotted on a glass coverslip and MDA-MB-231 cells were allowed to spread on this composite network for 15 min before to be fixed and stained for vinculin (red). Scale bar: 10 μ m. **d**, Quantification of the average vinculin staining fluorescence intensity on EGF-decorated and non-decorated (Naked) fibers (ns: non-significant, Student's t-test. N=3). Values for non-decorated fibers were set to 100%. All results are expressed as mean \pm SD.

Supplementary Figure 5. **Characterization of collagen fibers remodelling.** **a**, Genome-edited MDA-MB-231 cells expressing mCherry-tagged μ 2-adaptin (red) were seeded onto Alexa488-labelled collagen fibers (green) spotted on a glass coverslip for 1h and imaged by spinning disk microscopy by focusing on the dorsal surface of the cells. Scale bar: 10 μ m. **b**, Kymograph depicting the sequence of events occurring over time in the boxed area shown in **a**. **c**, Quantification of the average evolution of EGF-decorated (green) and non-decorated (Naked, red) collagen fibers circularity upon cell seeding on a composite 2D network as in Fig. 3d and when using cells treated with the indicated siRNAs or with Gefitinib (* P<0.01, Student's t-test. N=3). **d**, Quantification of the average cell velocity of MDA-MB-231 cells evolving in homogenous 3D networks composed of non-decorated or of EGF-decorated fibers, as indicated (ns: non-significant, Student's t-test. N=3). All results are expressed as mean \pm SD.

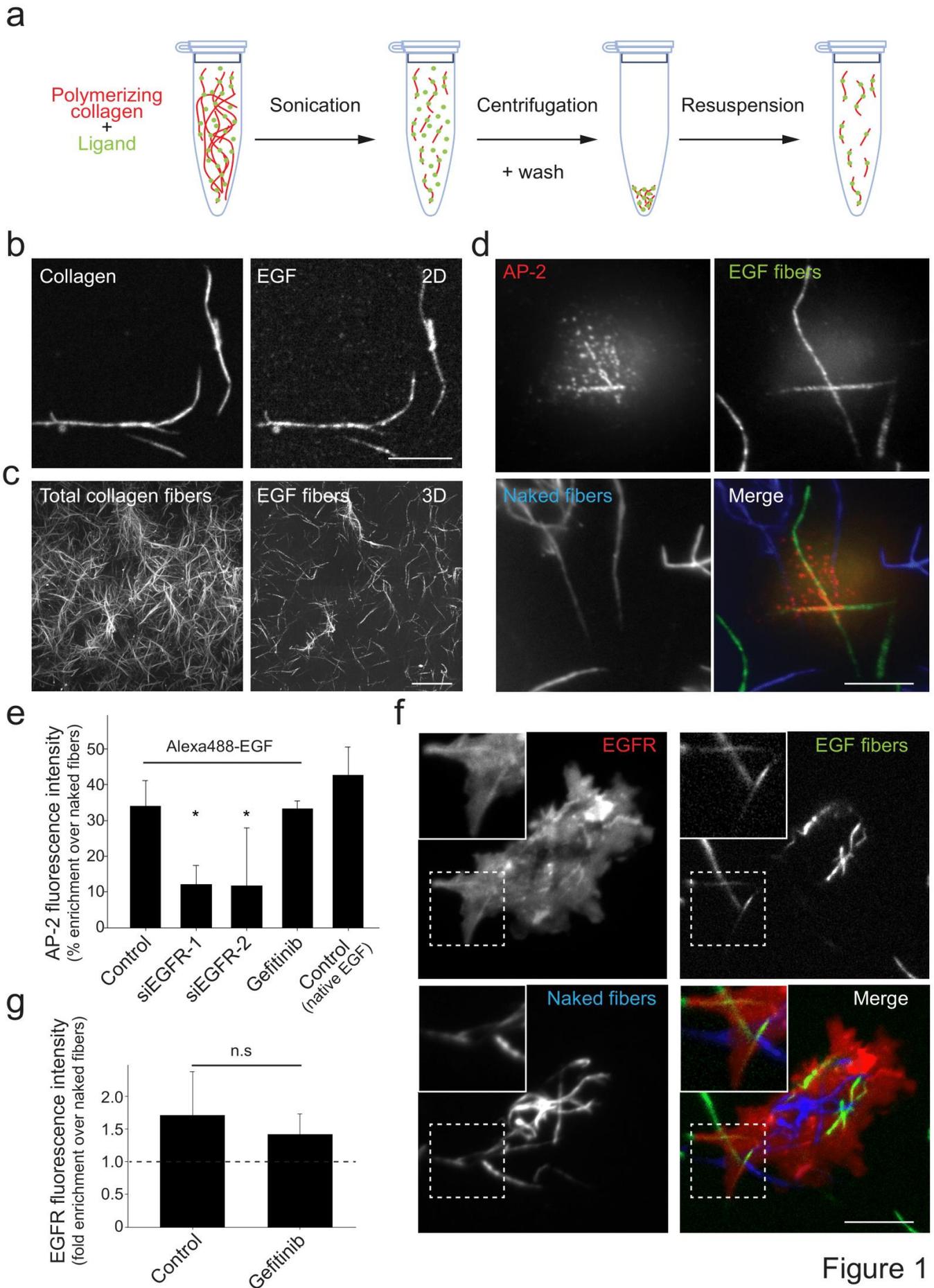


Figure 1

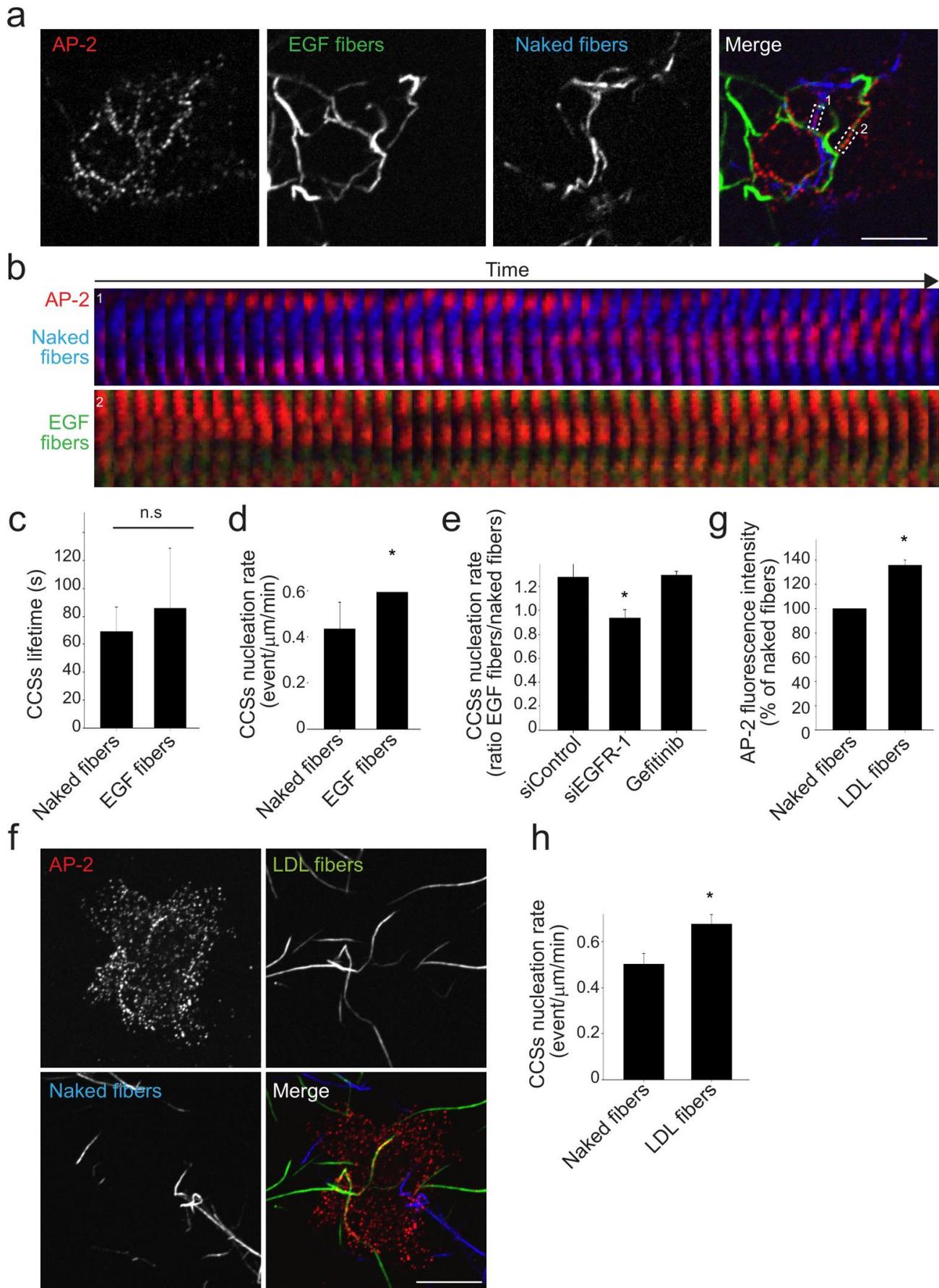


Figure 2

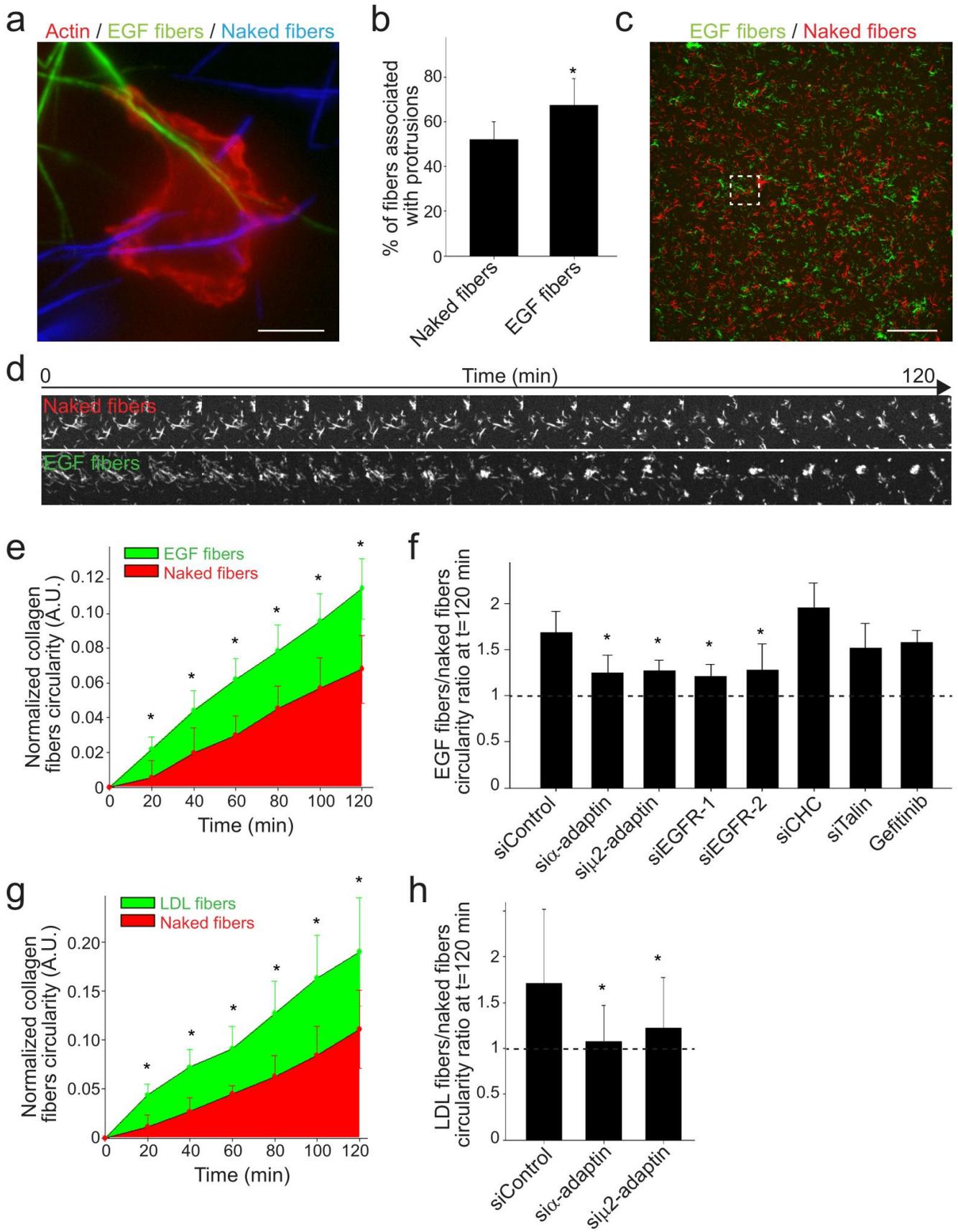


Figure 3

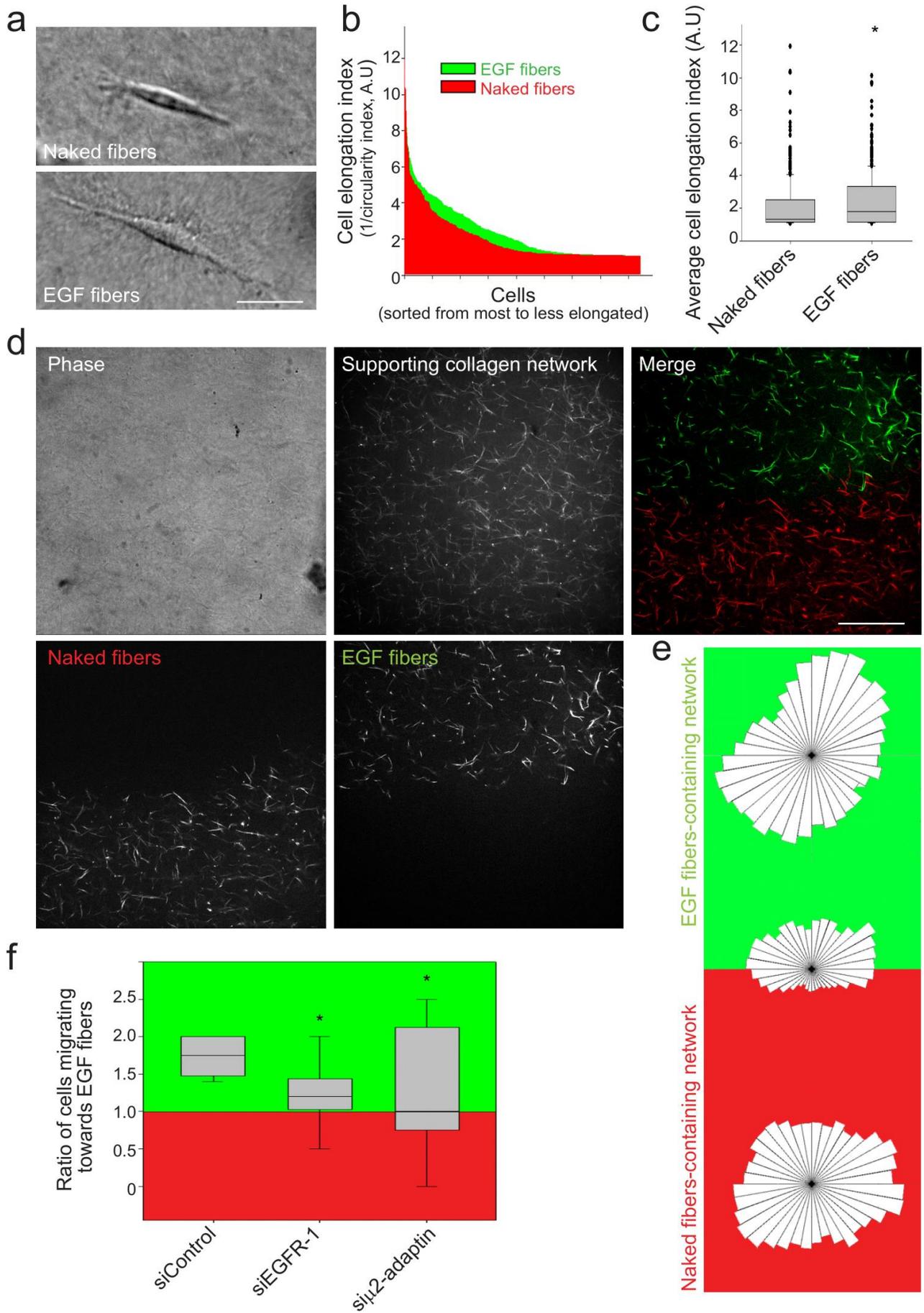


Figure 4

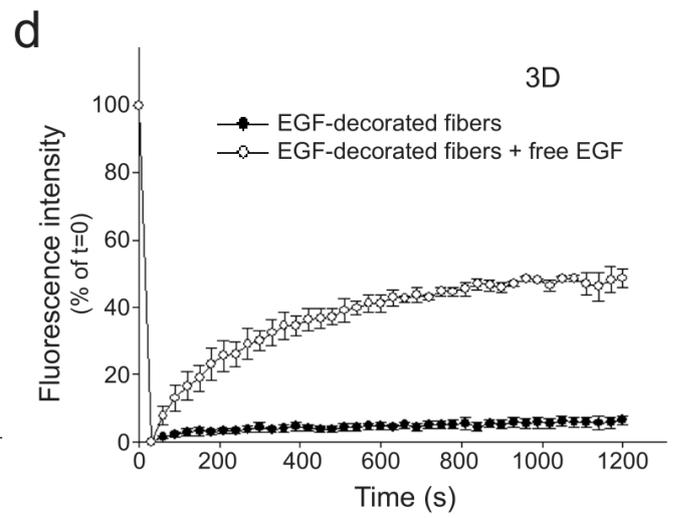
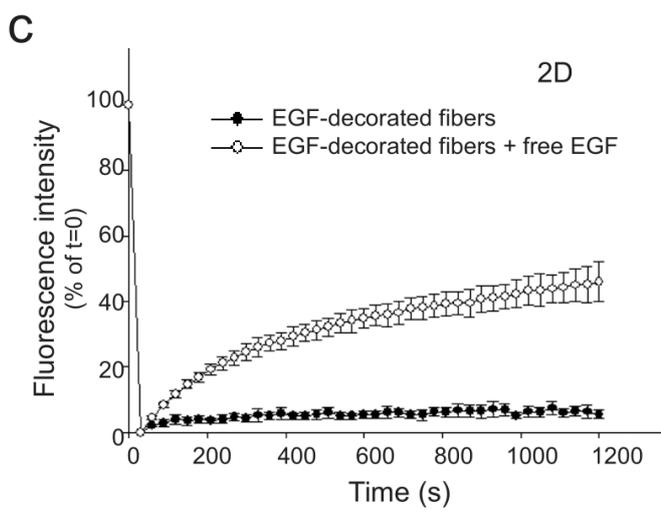
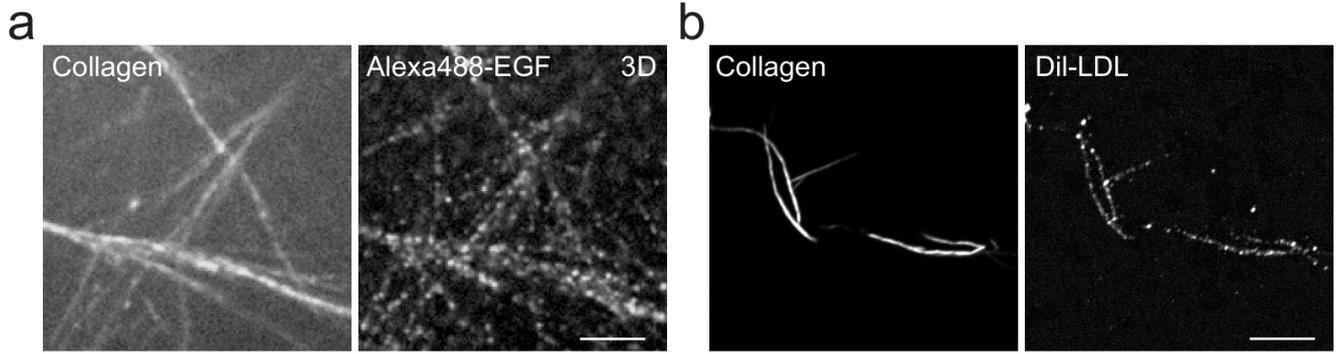


Figure S1

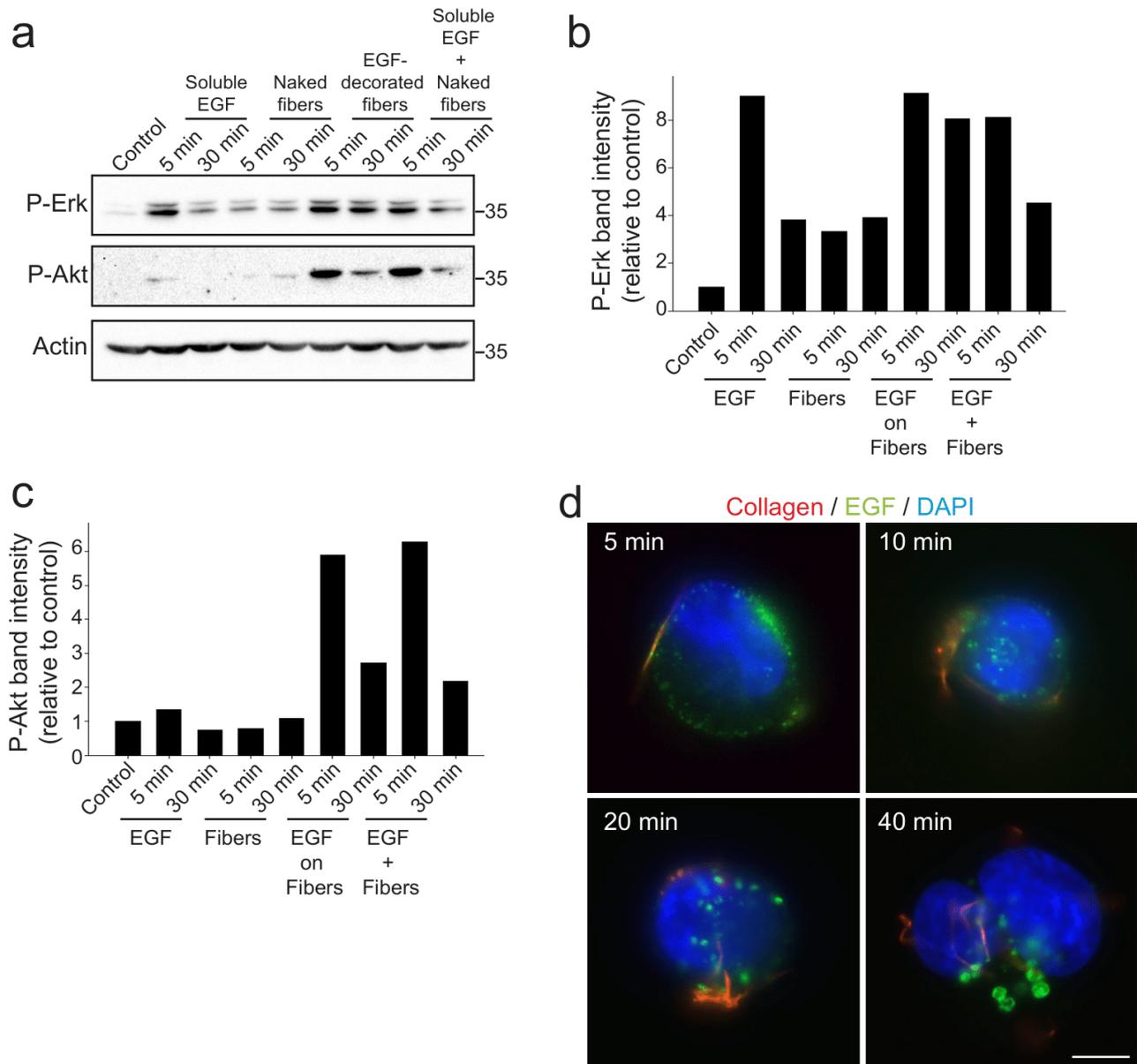
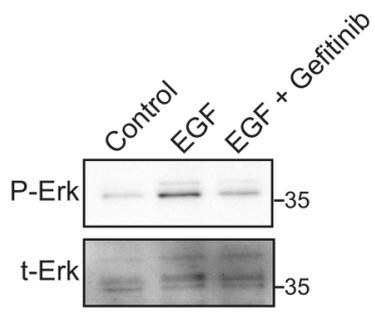
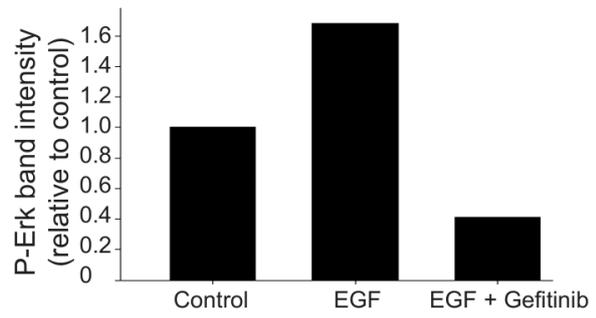
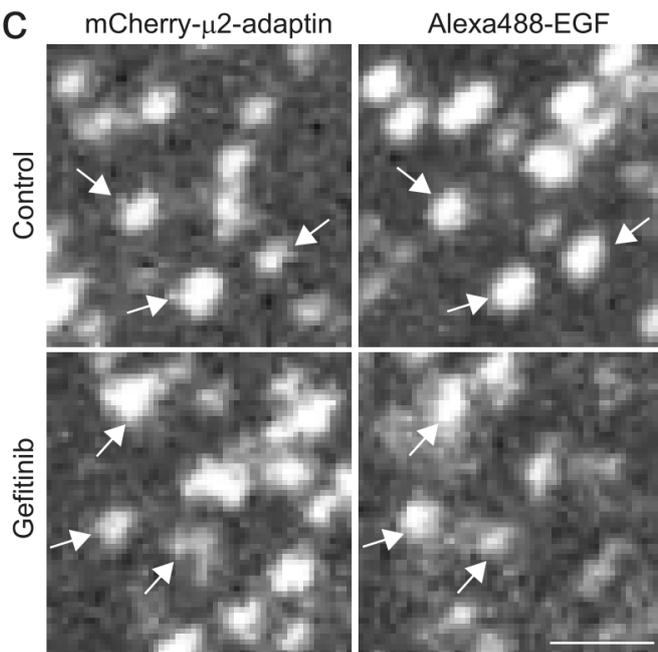
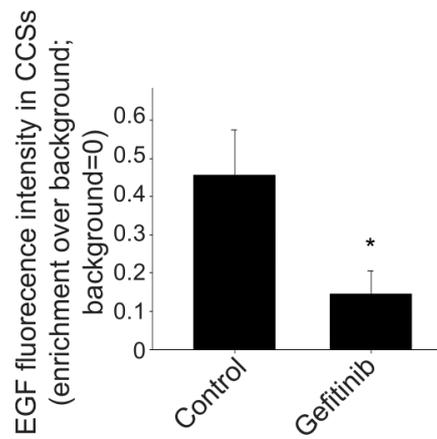


Figure S2

a**b****c****d****Figure S3**

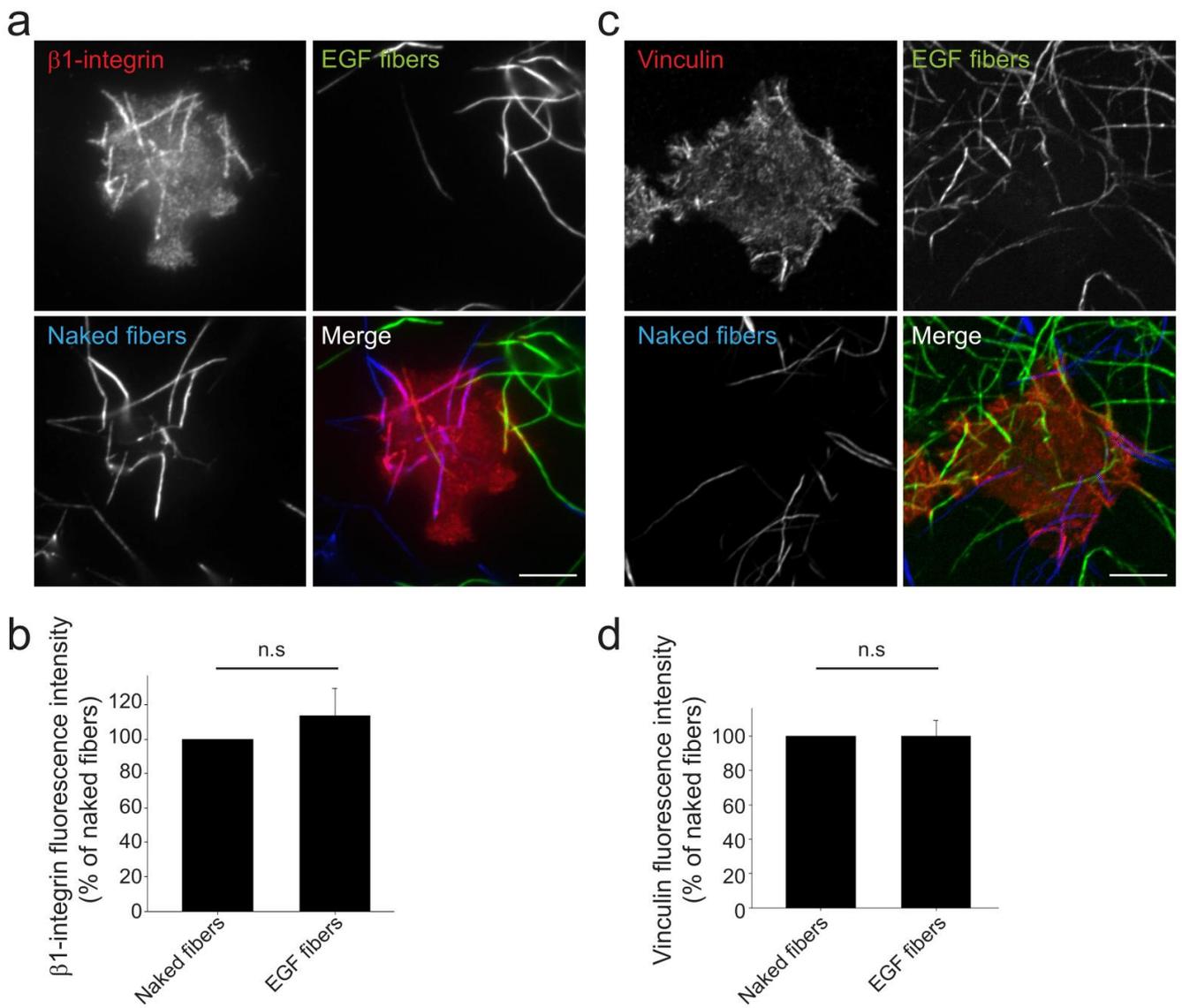


Figure S4

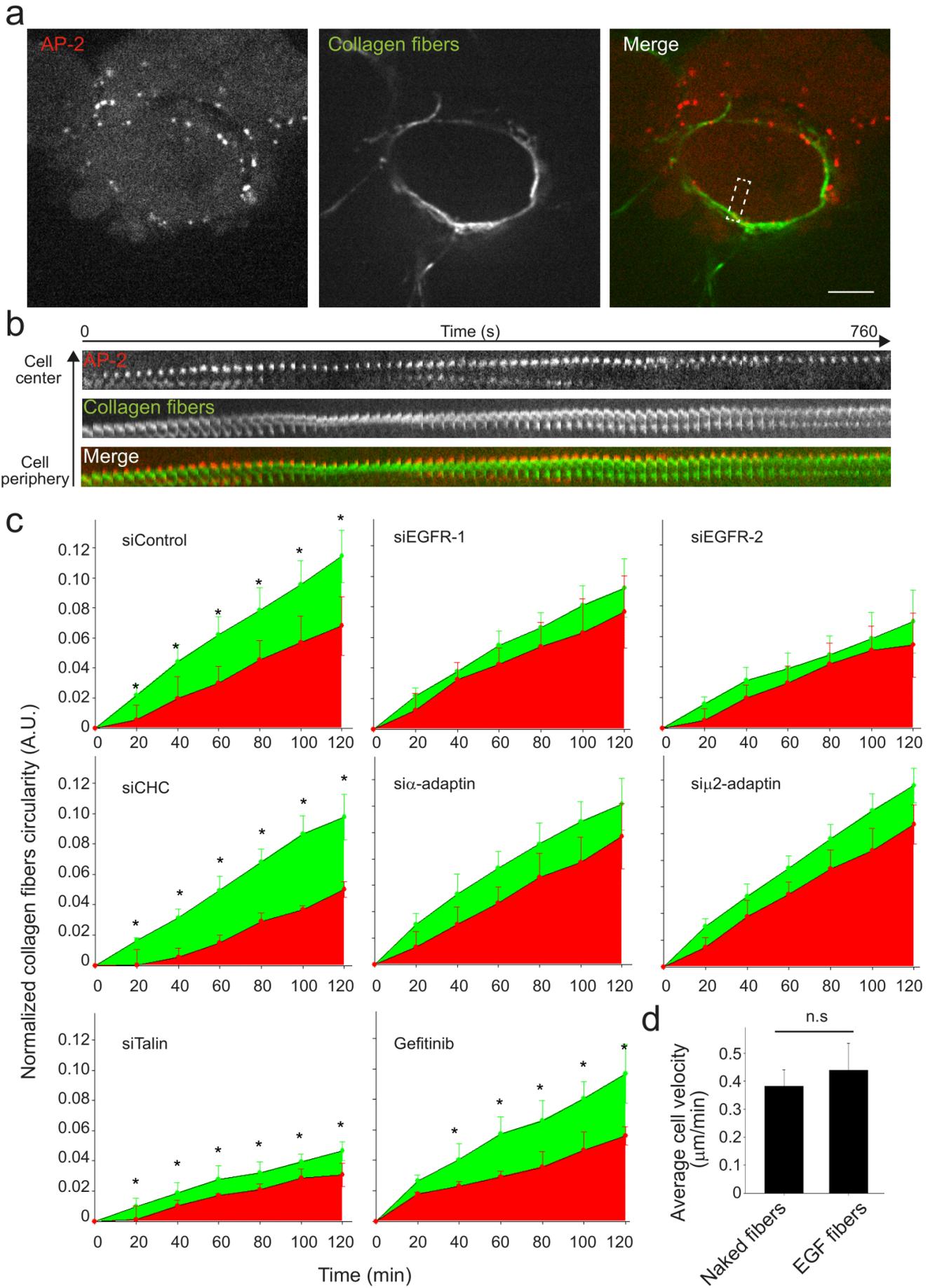


Figure S5

DISCUSSION

Cell movement is driven by polymerizing actin pushing the plasma membrane at the front of the cell. Actin polymerization can drive efficient migration only if the actin cytoskeleton is physically engaged with the extracellular matrix. Focal adhesions are the best documented adhesion structures. They cluster matrix receptors and link them to the actin cytoskeleton, thus playing a key role in migration.

We recently discovered that clathrin-coated structures (CCSs), previously known only as endocytic structures, can also serve as adhesion structures to collagen fibers. CCSs accumulate along collagen fibers because 1) CCSs nucleation is triggered by the curvature that the fiber imposes to the plasma membrane and 2) CCSs cluster $\beta 1$ integrin, a collagen receptor. These adhesive CCSs were termed tubular clathrin/AP-2 lattices (TCALs) because they adopt the tubular morphology of the collagen fibers, wrapping around and pinching the fibers.

During my PhD, I discovered a mechanism of 3D haptotaxis that relies on TCALs and local force transmission. I found that collagen fibers-bound CCS ligands are even more covered by TCALs than normal collagen fibers. This results from the preferential nucleation of CCSs on these fibers, leading to a local accumulation of adhesive structures which in turn leads to cell exerting more forces on ligand-decorated fibers as compared to non-decorated fibers. Finally, this mechanism is used by the cell to migrate directionally, by following matrix-bound protein in 3D networks.

1. - At least some CCS ligands can bind to collagen fibers

This project started with the observation that two CCS ligands (EGF and LDL) bind to *in vitro* polymerized collagen networks. These observations were already reported in the literature as EGF was shown to interact with monomeric collagen (Y. Yang et al. 2009) and electron microscopy imaging revealed that LDL can associate with collagen fibers in rabbit's cardiac valve (Nivelstein-Post et al. 1994). Because it is difficult to manipulate 3D collagen

network to address precise questions, I developed a protocol to produce short and manipulable collagen fibers and to decorate them with specific ligands.

Nothing is known about the mechanism of EGF or LDL interaction with collagen fibers as neither EGF nor LDL have known collagen binding domains. A study reported that the interaction of EGF with collagen display a K_d of 1.77×10^{-7} M (Kuo et al. 2015). This affinity seems weak but was measured using monomeric collagen and not with collagen assembled into fibers. It is possible that EGF have a much stronger affinity for polymerized collagen. Visualization of EGF-coated fibers were done using EGF labelled with the fluorophore Alexa488. Actually, Alexa488 is associated with streptavidin that binds biotin-decorated EGF. The Alexa488-streptavidin-biotin-EGF complex could impact on the apparent affinity/stability of EGF on collagen fibers as measured in my FRAP experiments. Indeed, more than 50% of EGF associated with collagen fibers was immobile, confirming the stability I observed. FRAP experiments on initially non-decorated collagen fibers in the presence of soluble EGF revealed that the strength of EGF/collagen interaction is essentially the same as for EGF-decorated fibers produce using my protocol. This rules out the possibility that EGF would better associate with polymerizing collagen than with already assembled fibers. Regarding LDL, nothing is known about its mechanism of binding to collagen and previous *in vitro* experiments revealed a poor binding of LDL to collagen (Pentikäinen et al. 1997). The observed LDL/collagen interaction have been proposed to be mediated through the collagen-binding ECM protein decorin. I however observed that LDL can bind to collagen fibers in the absence of any added factor.

Using cells transfected with fluorescent EGFR, I also confirmed that EGFR preferentially accumulate along EGF-decorated fibers as compared to normal collagen fibers. This accumulation is not affected by the EGFR inhibitor gefitinib. This is not surprising as gefitinib act through binding and inhibiting the kinase domain of the receptor that is not involved in EGF-EGFR interaction. In fact, gefitinib has been shown to increase EGFR affinity for its ligand (Björkelund, Gedda, and Andersson 2011) through unknown mechanisms.

2. - Collagen-bound EGF is active and can be internalized

I also observed that collagen-bound EGF could activate EGFR-dependent signaling pathways. Indeed, Erk was equally activated by similar concentrations of soluble or fiber-

associated EGF. However, while Erk can be activated by EGF alone in the experimental conditions I used (cells in suspension), Akt activation required the presence of both EGF and collagen fibers. This confirms the existence of crosstalk signaling between integrins and EGFR, at least for Ekt activation below EGFR signaling. These findings are in opposition with some results from the literature. For instance, Erk activation upon EGF stimulation was reported to be strongly decreased in non-adherent cells (Short, Talbott, and Juliano 1998). On the opposite, Akt was shown to be activated by EGF alone in cells in suspension (Velling, Stefansson, and Johansson 2008). These contradictory results may be explained by the different cell types used in these studies as compared to the MDA-MB-231 cells I used.

It also seems that Erk signaling is more sustained in time when EGF is associated with collagen fibers. This could result from the EGF/collagen interaction as the cell could take more time to internalize collagen-bound EGF, leading to a longer signaling period. This could also be due to the different pattern of EGF presentation to the cell. In one case, EGF is soluble and thus presented evenly distributed all around the cell. On the opposite, collagen fiber-bound EGF is presented as patches of local, high concentration and most of the rest of the membrane is free of EGF. In the case of soluble EGF, all receptors are internalized upon stimulation. When EGF is presented bound to collagen, most of the EGFR may remain available to sustain the signal. In any case, more precise kinetic experiments would be required to draw a definitive conclusion.

I also confirmed that cells internalize collagen-bound EGF. CCSs accumulation along EGF-decorated fibers suggest that collagen-bound EGF is directly internalized from the fibers. Nevertheless, it remains possible that a portion of EGF/EGFR complexes detach from the collagen fibers prior to be recruited at non-fiber associated CCSs. Here also, more experiments would be required to unambiguously demonstrate that CCSs that accumulate on fibers are actually able to internalize fiber-associated EGF.

3. - Accumulation of TCALs along collagen fibers decorated with CCS ligands

I observed that TCALs accumulate more on EGF/LDL-decorated collagen fibers as compared to non-decorated ones. The accumulation of TCALs along EGF-decorated collagen fibers is lost in cells depleted for EGFR but not in cells treated with gefitinib. TCALs preferential accumulation along ligand-coated fibers could result from an increase of either

CCS local nucleation rate and/or lifetime. I observed that CCS lifetime was only slightly increased on EGF-coated fibers. On the opposite, CCS nucleation rate was significantly increased along ligand-decorated collagen fibers as compared to naked fibers. EGFR depletion but not gefitinib treatment abolished the increased CCS nucleation rate on EGF-coated fibers. These observations could bring new insights on two ongoing debates: the requirement of EGFR phosphorylation for its internalization and the ability of receptors to mediate *de novo* formation of CCSs.

First, the requirement of EGFR phosphorylation for its recruitment into CCSs, and hence its internalization, remains unclear in the literature. Some studies found that the tyrosine kinase activity is required for EGFR recruitment at CCS (Sorkina et al. 2002) and internalization (Lamaze and Schmid 1995). Other studies suggested that receptor dimerization is sufficient for EGFR internalization, regardless of the phosphorylation state (Q. Wang, Villeneuve, and Wang 2005). Finally, a study found that artificial clustering of EGFR is sufficient to induce internalization (Heukers et al. 2013). In our case, gefitinib treatment did not affect the preferential accumulation of TCALs along EGF-coated collagen fibers nor the recruitment of EGF into CCSs. This suggests that EGFR tyrosine kinase activity, and hence autophosphorylation, is not required for EGFR recruitment at CCSs and subsequent internalization. This means that EGFR signaling and endocytosis are uncoupled, EGF-induced dimerization of its receptors regulating both signaling and endocytosis independently.

Second, the ability of receptors, especially EGFR, to induce *de novo* formation of CCSs is also debated. Some studies found that EGFR can induce CCSs nucleation in a signaling dependent manner (Johannessen et al. 2006; Wilde et al. 1999). On the other side, some observed that EGFR is internalized through preformed CCSs (Rappoport and Simon 2009). If the constitutively internalized LDLR was never reported to induce *de novo* formation of CCSs, a study found that clustering of the constitutively internalized transferrin receptor induces CCS nucleation (A. P. Liu et al. 2010). In our case, collagen fiber-bound ligands induce CCS nucleation using a signaling independent mechanism as gefitinib did not affect this parameter, and LDLR is not known to elicit any specific signaling event. Either the observed EGF/LDLR-induced nucleation relies on different mechanisms or they rely on similar mechanisms potentially induced by their association with collagen fibers. For EGFR, we can hypothesize that dimerization reveals a functional endocytosis motif, as suggested, that further recruits the endocytic machinery, leading to CCS formation. Another non-exclusive and very likely

hypothesis is that the accumulation of EGF or LDL along collagen fibers induce the clustering of their receptors. These clustered receptors could induce CCS formation as reported for Tfr in experimental clustering conditions (A. P. Liu et al. 2010).

4. - Cells protrude more on EGF-coated collagen fibers

I observed that cells tend to protrude more on EGF-decorated fibers spotted on 2D coverslips. Around 70% of EGF-coated fibers were associated with a protrusion and this proportion drop to 50% for non-coated fibers. Protrusion formation is driven by actin polymerization and EGF ability to locally induce actin polymerization (Kempiak et al. 2003) could explain the preferential protrusion activity on EGF-decorated fibers. Another possibility is that the preferential protrusion activity on EGF-coated collagen fibers is linked to the preferential CCSs accumulation on these fibers (see next section). On the opposite, neither integrins nor focal adhesions seemed to be involved in this mechanism as they distributed evenly between naked and EGF-coated collagen fibers. Further experiments are required to determine the actors and mechanisms involved in preferential protrusion along EGF-coated collagen.

5. - Cells pulls more on CCS ligands-decorated collagen fibers

I observed that when cells are plated on the top of collagen fibers sparsely spotted on a coverslip, they pull on the fibers and pack them on their dorsal surface as condensed aggregates. We took advantage of this to estimate cells remodeling capacities by measuring the evolution over time of fibers circularity. I observed that cells preferentially remodel collagen fibers that are decorated with CCS ligands as compared to naked fibers.

In the case of both EGF- and LDL-decorated fibers, AP-2 depletion reduced the preferential remodeling. This means that more forces are applied on decorated-fibers in a CCSs-dependent manner. However, although the preferential remodeling is lost in these conditions, it was not the consequence of a reduced remodeling of decorated fibers but was rather due to an increased remodeling of non-decorated ones. Observations from the lab showed that focal adhesions are increased in size upon CCSs depletion, probably reflecting the role of endocytosis in regulating the dynamics of these structures. This could explain the overall increase in remodeling levels observed after AP-2 depletion. However, clathrin depletion did not reduce

the preferential remodeling of EGF-coated fibers. This is in agreement with our previously published model in which TCALs role as adhesive structures were actually shown to be independent of clathrin. In the absence of clathrin, a structural core made of AP-2 and other adaptors remains, still able to cluster receptors, and still able to wrap around and pinch collagen fibers (Elkhatib et al. 2017). In addition, the fact that clathrin depletion did not inhibit the preferential remodeling of decorated fibers indicate that this process is independent of endocytosis and probably rely only on the adhesive role of TCALs as we previously reported.

EGFR depletion also abolished the preferential remodeling of EGF-coated fibers. Decorated fibers were specifically less remodeled in these conditions as compared to control cells. This confirms the role of EGFR in the preferential remodeling of EGF-coated fibers, most likely through its role in promoting local TCALs nucleation on decorated fibers. In agreement with my observations that tyrosine kinase activity is not involved in TCALs nucleation on EGF-decorated fibers, its inhibition using gefitinib did not affect the preferential remodeling of these fibers. The observed preferential remodeling of LDL-coated fibers also advocates for a signaling-independent mechanism.

Finally, talin depletion did not affect the preferential remodeling of EGF-decorated fibers. However, the global levels of remodeling were strongly reduced. This suggests that focal adhesions play an important role in fibers remodeling but do not allow to discriminate between the different types of fiber, in agreement with my observations that focal adhesions are equally distributed between decorated and non-decorated fibers.

We already reported that TCALs help the cell to grab and move collagen fibers. My data here suggest that TCALs are responsible for the increased forces applied on decorated fibers. Force production in the cell is usually provided by the actin network but it is not known if and how TCALs can connect actin to transmit forces. Focal adhesion directly interact with the actin network and this slows down the actin retrograde flow, leading to effective pushing forces and generation of protrusions. A similar mechanism could be at play here. Observations from the lab show that, in cells crawling on a 2D surface, CCSs located at the leading edge move toward the cell center before budding and actually follow the actin retrograde flow. This means that they are somehow connected to the actin cytoskeleton. Several components found in CCSs are able to bind actin and thus, CCSs anchored to the substrate could potentially slow down the actin retrograde flow. It is also possible that actin interacts more sterically than specifically with

the TCALs/CCSs. The actin retrograde flow could simply bump into the membrane invagination formed by CCSs or bump into the fibers upon TCALs-mediated membrane wrapping around fibers. In any case our data suggest that the more TCALs on a fiber, the more forces are applied on this fiber and thus more forces are applied on CCS ligands-decorated fibers.

6. - In 3D, cells migrate towards CCS ligands-decorated collagen networks

To see if collagen-bound CCSs ligands affect 3D adhesion and migration, naked or decorated collagen fibers were incorporated into 3D collagen networks. Cells were more spread in EGF-decorated 3D collagen network as compared to regular collagen network. The increased spreading could be due to more TCALs accumulating on EGF-decorated fibers and facilitating cell adhesion to surrounding fibers. This still needs to be confirmed by ablating TCALs formation. Yet, cell velocity was not increased in EGF-decorated networks. This may reflect a global stabilization of protrusions pointing in different direction so that *in fine* no net advantage for motility can be measured in these conditions. However, when at the interface between regular and EGF-decorated networks, cells preferentially migrated towards the EGF-decorated network. This directed migration was lost upon depletion of EGFR or AP-2. These findings reveal an EGF-dependent haptotaxis in 3D that relies on clathrin structures.

At the interface, cells most likely protrude in random directions, but it is likely that the extra accumulation of TCALs along EGF-decorated fibers may stabilize protrusions pointing towards these types of fibers. As a consequence, cells would preferentially migrate towards these fibers, leading to the directed migration I observed. Additional experiments on protrusion lifetime at the interface are required, though, to test and validate this model.

7. - Model and conclusion

Together, my results allow to draw a general model for haptotaxis mediated by CCS ligands. Asymmetric distribution of fiber-associated CCS ligands around the cell leads to an asymmetrical distribution of adhesive TCALs because of local increased nucleation rate on ligands-decorated fibers. Because we previously reported that TCALs help to stabilize protrusions, this asymmetric distribution of TCALs may lead to an asymmetric stabilization of protrusions and an asymmetric distribution of forces applied on the ECM. This mechanism

would induce a directed migration towards ECM-anchored CCS ligands. Interestingly, the local accumulation of TCALs that drives this 3D haptotaxis does not rely on activation of signaling pathways as proposed for classical chemotaxis.

These results suggest that haptotaxis is mediated by different mechanisms from chemotaxis, at least in the conditions used in this study. In the case of EGF, chemotaxis is mediated by an asymmetric distribution of actin polymerization in response to EGFR signaling. In our model, EGF haptotaxis is driven by an asymmetric distribution of TCALs, independently of EGFR signaling. *In vivo*, directed migration towards EGF gradients is probably a combination of both mechanisms.

I here provide evidences for haptotaxis mediated by EGF or LDL, but the described mechanism could apply to any CCS ligands able to bind to the ECM. Many CCS ligands are known to associate with collagen or with other ECM components. 3D haptotaxis mediated by TCALs could thus be found in numerous examples of directed migration *in vivo*, from embryogenesis to cancer invasion.

In addition I set up a protocol allowing the study of haptotaxis independently from chemotaxis. The effect of decorated-collagen fibers can be tested in cell in suspension, in 2D or in 3D collagen networks. This allows to study the induced signaling activation, to visualize the localized effect of decorated fibers and to test the ability of collagen-bound chemoattractants to induce directed migration. This protocol could be used for other CCS ligands but also to decorate collagen fibers with some ECM components in order to build and assemble complex network in a precise manner. In conclusion, this protocol may prove useful for many studies in different fields.

Tubular clathrin/AP-2 lattices pinch collagen fibers to support 3D cell migration

Nadia Elkhatib, **Enzo Bresteau**, Francesco Baschieri, Alba López Rioja, Guillaume van Niel, Stéphane Vassilopoulos and Guillaume Montagnac

Science. 2017 Jun 16;356(6343). pii: eaal4713. doi: 10.1126/science.aal4713.

RESEARCH ARTICLE

CELL ADHESION

Tubular clathrin/AP-2 lattices pinch collagen fibers to support 3D cell migration

Nadia Elkhatib,^{1*} Enzo Bresteau,¹ Francesco Baschieri,¹ Alba López Rioja,¹ Guillaume van Niel,^{2†} Stéphane Vassilopoulos,³ Guillaume Montagnac^{1*}

Migrating cells often use focal adhesions in order to move. Focal adhesions are less prominent in cells migrating in three-dimensional (3D) as compared with 2D environments. We looked for alternative adhesion structures supporting cell migration. We analyzed the dynamics of clathrin-coated pits in cells migrating in a 3D environment of collagen fibers. Both topological cues and local engagement of integrins triggered the accumulation of clathrin-coated structures on fibers. Clathrin/adaptor protein 2 (AP-2) lattices pinched collagen fibers by adopting a tube-like morphology and regulated adhesion to fibers in an endocytosis-independent manner. During migration, tubular clathrin/AP-2 lattices stabilized cellular protrusions by providing anchoring points to collagen fibers. Thus, tubular clathrin/AP-2 lattices promote cell adhesion that, in coordination with focal adhesions, supports cell migration in 3D.

Clathrin-mediated endocytosis is a fundamental process that controls a wide variety of cell functions, including cytokinesis (1), cell migration, and cell invasion (2). Clathrin-coated structures (CCSs) allow migrating cells to adapt to their environment by selectively controlling the uptake of specific cargos (3), regulating processes such as cell protrusion (4) and adhesion dynamics (5, 6). CCS recruitment at focal adhesions (FAs) regulates integrin endocytosis and leads to adhesion disassembly and cell movement (5, 6). Conversely, CCS dynamics is locally modulated by cellular adhesions (7, 8). However, whether this reflects a physical engagement of integrin-rich CCSs with the extracellular matrix (ECM) or an indirect regulation by adhesion sites is unclear. In addition, integrin-positive flat clathrin structures (9) establish tight contacts with planar substrates (10), suggesting that they could participate directly in cell adhesion (11). The extracellular environment impinges on adhesion dynamics, and FAs are reduced in size and number when cells are cultured on physiologically soft substratum or in a soft three-dimensional (3D) network of collagen fibers (12–16). However, although clathrin-mediated endocytosis plays a role in cancer cell migration in a 3D network of collagen fibers (4, 17), CCS dynamics, structure, and functions in physiologically relevant environments are unknown. We set out to investigate CCS dynamics in cells

facing a 3D environment composed of collagen fibers.

CCSs accumulate along collagen fibers

We first observed that CCSs of breast cancer-derived MDA-MB-231 cells marked with the α -adaptin subunit of the clathrin adaptor protein 2 (AP-2) were often located along collagen fibers in the 3D network (Fig. 1A). Visualization of 3D reconstructions of cells in the collagen network (movie S1), followed by automatic detection of CCSs and fibers, revealed that $63.3 \pm 6.6\%$ (SEM) of CCSs were in contact with fibers [as compared with $43.5 \pm 7.9\%$ (SEM) when the position of collagen fibers were randomized; $P < 0.01$] (Materials and methods). Electron microscopy analyses confirmed that CCSs established intimate contacts with collagen fibers [$64.1 \pm 17.5\%$ (SEM) of detectable CCSs were contacting collagen fibers] (fig. S1, A to C). Although the ultrathin sectioning of the sample did not allow us to fully visualize the morphology of CCSs in contact with fibers, we occasionally observed collagen fibers apparently laying in the cleft of invaginated CCSs (Fig. 1B and fig. S1, A to C). The strong accumulation of CCSs along collagen fibers suggested that either the lifetime of CCSs and/or their nucleation rate at cell/collagen fibers contact sites were increased. Live cell imaging of genome-edited MDA-MB-231 cells expressing a green fluorescent protein (GFP)-tagged endogenous μ 2-adaptin subunit of AP-2 (18) revealed that CCSs tended to nucleate preferentially on collagen fibers (Fig. 1C, fig. S1D, and movie S2) and that fiber-contacting CCSs were longer-lived as compared with CCSs located in other regions of the plasma membrane (Fig. 1D and movie S2). These observations explain why CCSs accumulate on collagen fibers and suggest that collagen fibers control CCS recruitment.

To study the underlying mechanisms of this process, we used a 2D system by polymerizing a limited number of collagen fibers on glass coverslips. Cells plated on such a substratum contact both collagen fibers and the collagen-coated glass coverslip. Fifteen min after plating cells, CCSs strongly aligned along collagen fibers (Fig. 1E). Similar accumulation of CCSs along collagen fibers was also visible in HeLa cells (fig. S2A). Vinculin-positive focal and/or nascent adhesions were often found on collagen fibers as well but were more peripherally distributed as compared with CCSs, and CCSs did not colocalize with them nor with cortactin stretches that mark ECM degradation structures termed linear invadopodia (fig. S2, B and C) (19, 20). In addition, clathrin heavy chain (CHC) and dynamin-2 were not required for AP-2-positive structure recruitment on collagen fibers (fig. S2, D to G). When cells were allowed to spread for more than 15 min, the number of CCSs colocalizing with collagen fibers dropped, and by 1 hour, no obvious alignment was detected anymore (Fig. 1E). The loss of CCSs from collagen fibers during cell spreading on 2D differs from the 3D condition in which CCSs were consistently seen associated with fibers long after embedding the cells in the collagen network. We noticed that cell spreading area was inversely correlated with the degree of CCSs alignment along fibers at any given time point after cell plating on 2D (Fig. 1F). Inhibiting cell spreading by inactivating the FA-associated protein talin increased alignment of CCSs along collagen fibers, also at late time points (fig. S2, G to I). Because membrane tension increases during cell spreading in two dimensions (21, 22) and has been proposed to be weaker in softer 3D environments (23), we speculated that it could be an important parameter regulating CCS accumulation on collagen fibers. Indeed, increasing membrane tension by exposing cells to hypotonic medium led to a partial loss of CCSs from fibers (Fig. 1G). Thus, high membrane tension resulting from cell spreading on a stiff 2D substrate prevents CCS accumulation on collagen fibers.

Tubular clathrin/AP-2 lattices pinch collagen fibers

We next took advantage of the 2D system to analyze the morphology of collagen fiber-contacting CCSs in a more comprehensive manner than was possible in the 3D condition. For this, we performed metal-replica electron microscopy analysis of the adherent plasma membrane of unroofed cells after a 15-min plating period onto collagen fibers. The electron density of collagen fibers made them easily traceable, even when located below the plasma membrane (Fig. 2, A to F). This feature allowed us to unambiguously analyze “en face” the many CCSs that were detected on collagen fibers, which is in agreement with our immunofluorescence analyses (Fig. 2, A to D). Clathrin coats on fibers were composed of continuous and interconnected lattices that elongated along the long axis of the fiber, seemingly wrapping around and pinching it (Fig. 2, B to D, and fig. S3, A and B). 3D visualization confirmed that these structures assembled as semitubes around collagen fibers (Fig. 2, C and

¹Inserm U1170, Gustave Roussy Institute, Université Paris-Saclay, Villejuif, France. ²Institut Curie, UMR144, Université de Recherche Paris Sciences et Lettres, Centre Universitaire, Paris, France. ³Inserm/Université Pierre et Marie Curie UMR S974, Institut de Myologie, Paris, France.

*Corresponding author. Email: guillaume.montagnac@gustaveroussy.fr (G.M.); nadia.elkhatib@gustaveroussy.fr (N.E.)
†Present address: Center of Psychiatry and Neurosciences, U894 INSERM, Paris, France.

D, and fig. S3, A and B, insets), demonstrating the plasticity of clathrin assemblies that were able to adopt the topology of the plasma membrane. These tubes could span the size of three to four canonical clathrin-coated pits and may represent a curved version of the flat clathrin lattices observed in areas of the plasma membrane far from collagen fibers (Fig. 2C and fig. S3B). It is also possible that clathrin-coated tubes are an intermediate arrangement of maturing CCSs on fibers. Furthermore, we confirmed through immunogold labeling of cells completely lacking any detectable clathrin coats at the plasma membrane (Materials and methods) that CHC was dispensable for the accumulation of α -adaptin on collagen fibers (Fig. 2E and fig. S3C). Close inspection of metal replicas revealed the presence of characteristic patches of proteinaceous material, which corresponded to the coat of clathrin adaptors (Fig. 2, G to I, and fig. S3, D and E). These structures were also found to accumulate on collagen fibers, confirming that clathrin itself was not required for patches of adaptors to accumulate on and to wrap around fibers (Fig. 2F). Thus, we named the CCSs that accumulated on collagen fibers tubular clathrin/AP-2 lattices (TCALs).

In agreement with our immunofluorescence analysis, we observed fewer clathrin coats on collagen fibers after a 30-min plating period (fig. S4A). In contrast, and also confirming our immunofluorescence analysis, TCALs were abundant on collagen fibers in talin-depleted cells after a 30-min spreading period (fig. S4, B to D). Flat clathrin lattices in regions away from fibers were rare in control cells at this time point (fig. S4A). This latter observation is in agreement with the measured lifetime of CCSs in cells plated on a planar, collagen-coated substratum and analyzed during different time windows upon plating [69.2 ± 28.8 s (SEM) during the first 15 min of spreading versus 43.2 ± 13.9 s (SEM) between 15 and 30 min of spreading], suggesting that clathrin structures are longer-lived early after plating because they remain flat for a prolonged period of time.

Mechanism of CCS recruitment on fibers

Both CCS lifetime and nucleation rate are increased on collagen fibers in the 3D network, explaining why CCSs accumulate on fibers (Fig. 1, C and D). However, in the 2D system we did not measure a significant difference in lifetime between CCSs located on fibers or in other areas of the plasma membrane (Fig. 3A). This may have been a consequence of CCSs' engagement with the ECM because in this assay, the cell contacted both collagen fibers and the collagen-coated glass. Nevertheless, CCS nucleation rate was increased on collagen fibers in the 2D system, which is in agreement with our observations in the 3D situation (Fig. 3, B and C). We reasoned that the plasma membrane of cells in contact with collagen fibers is likely to be locally curved. Indeed, adaptor-dependent clathrin-assembly is facilitated by membrane curvature in reconstituted *in vitro* systems (24). Thus, we decided to analyze whether the local topology of the plasma membrane could account for an increased CCS nucleation rate. To test this hypothesis, we plated cells onto noncoated fluorescent beads

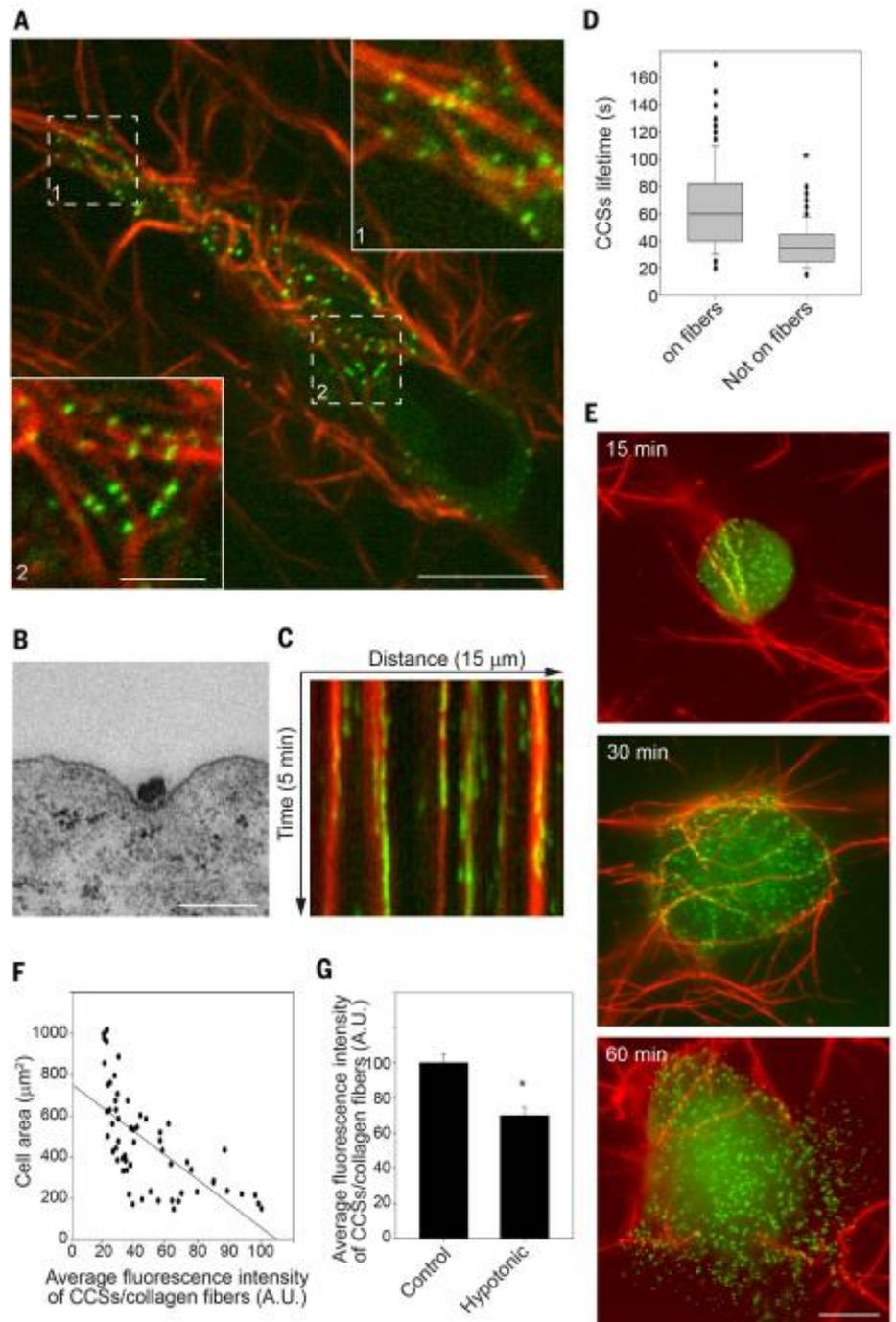


Fig. 1. CCSs accumulate along collagen fibers. (A) MDA-MB-231 cells were seeded in a 3D network of collagen fibers (red) and fixed 24 hours later before being stained for α -adaptin (green). A single optical section is shown. Scale bar, 10 μ m. (Insets) Higher magnifications of boxed regions. Scale bars, 3 μ m (B) Electron microscopy micrograph of a MDA-MB-231 cell in the 3D collagen network depicting a collagen fiber in the cleft of a CCS. Scale bar, 100 nm. (C) Kymograph showing CCS (green) dynamics and collagen fibers (red) in a genome-edited MDA-MB-231 cell expressing endogenous GFP-tagged μ 2-adaptin, imaged with spinning disk microscopy every 5 s for 5 min in the 3D collagen network. (D) Quantification of the lifetime of CCSs located on fibers or not, as indicated in genome-edited MDA-MB-231 cells in the 3D network (mean \pm SEM; $n \geq 125$ CCSs, $*P < 0.01$, Student's *t* test). (E) MDA-MB-231 cells spreading on a thin layer of collagen fibers (red) polymerized on glass were fixed at the indicated time point and stained for α -adaptin (green). (F) Distribution of the average fluorescence intensity of α -adaptin staining per collagen fibers as a function of cell spread area. For linear regression, correlation coefficient (r) = 0.6475; $P < 0.0001$, ANOVA test. (G) Average fluorescence intensity of α -adaptin staining on collagen fibers 15 min after plating in control or hypotonic medium as indicated (mean \pm SEM; $n = 3$ experiments, $*P < 0.01$, Student's *t* test).

spotted onto collagen-coated glass coverslips. We used 200-nm-diameter beads to mimic the deformation imposed by collagen fibers that have a comparable diameter. At 15 min after plating, CCSs often colocalized with fluorescent beads (Fig. 3, D and E). The number of beads colocalizing with CCSs was higher than expected by chance because randomizing the position of beads dramatically reduced the occurrence of colocalization events (Fig. 3E). Live cell imaging analysis showed that beads were stably bound to the coverslip and were not internalized by cells (Fig. 3F). In addition, multiple CCSs sequentially assembled on the same beads, confirming that local membrane curvature triggers CCS nucleation (Fig. 3F). When using collagen-coated fluorescent beads, the percentage of beads colocalizing with CCSs increased to nearly 45% (Fig. 3E). This suggested an active and local role of the ECM in CCS accumulation at curved sites. Because membrane curvature is key in controlling local CCS nucleation, and membrane tension inhibits CCS accumulation on collagen fibers (Fig. 1G), it is possible that high tension prevents the membrane to bend sufficiently at fiber/cell con-

tact sites, thus preventing an increased CCS nucleation rate. Accordingly, we observed a slight but reproducible defect of CCS accumulation on beads when membrane tension was increased upon incubation in hypotonic medium (Fig. 3G).

β 1-integrin (the β -subunit of all the human collagen-binding integrin heterodimeric receptors) strongly accumulated along the entire length of collagen fibers in contact with the cell in the 2D assay, which is in agreement with recent findings in 3D collagen networks (Fig. 4A) (25). However, close inspection of β 1-integrin staining showed that it was enriched at CCSs (Fig. 4B and fig. S5A). In addition, β 1-integrin was also enriched at a subset of CCSs in cells plated on a planar, collagen-coated coverslip (fig. S5B). The alternative clathrin-adaptor Dab2 has been proposed to link β 1-integrins to CCSs (5, 26–28), and we observed that the vast majority of CCSs, including those located on collagen fibers, contained Dab2 (fig. S5C). Dab2 silencing inhibited β 1-integrin enrichment in CCSs located on fibers, suggesting that it is necessary to cluster integrins at CCSs (Fig. 4C). In addition, cells depleted for Dab2 did not display the strong

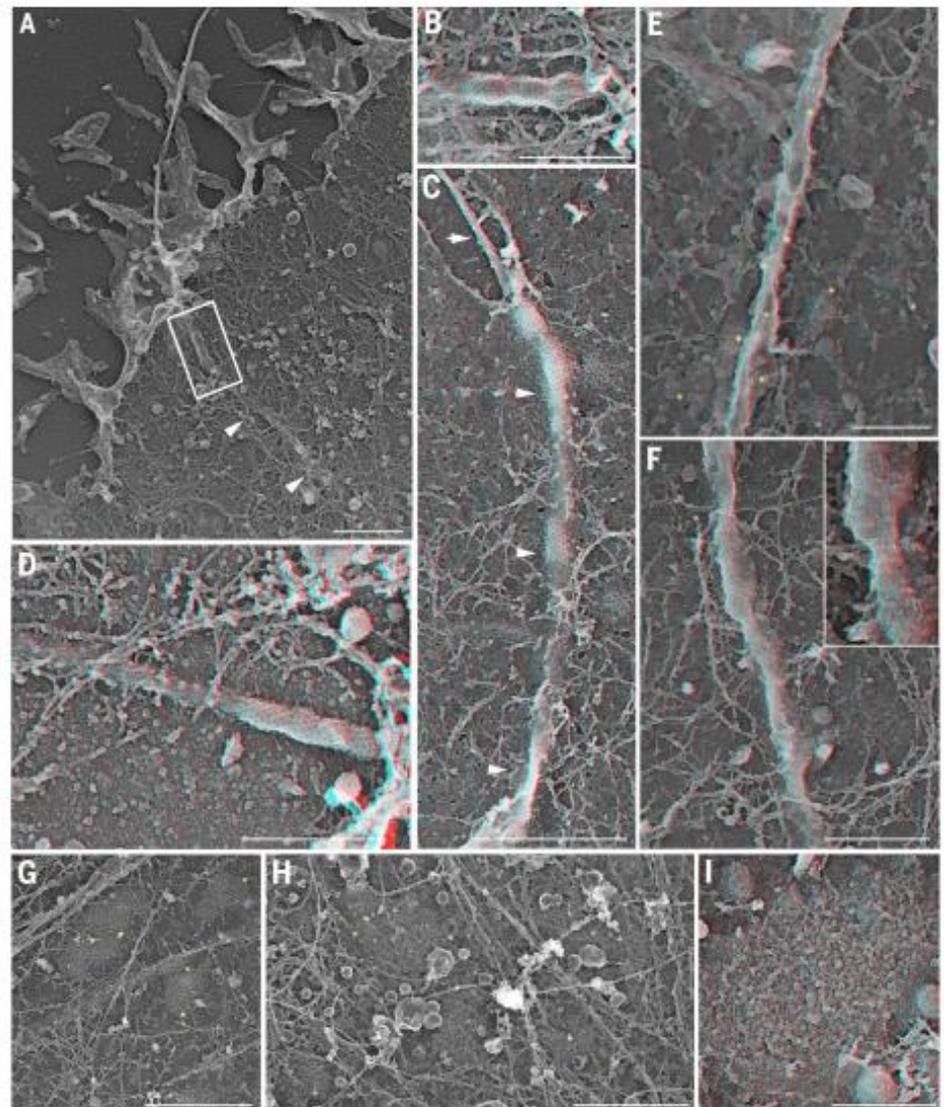
accumulation of CCSs along collagen fibers that is observed in control cells after a 15-min plating period (Fig. 4D and fig. S6, A to D). It was not possible to directly assess the role of β 1-integrin in CCSs recruitment on collagen fibers in the 2D system because its inhibition prevents cell adhesion. However, inhibiting β 1-integrins by using a blocking antibody (4B4) significantly reduced the lifetime of fibers-associated CCSs in the 3D network, without affecting the lifetime of CCSs in other region of the plasma membrane (Fig. 4E). Similarly, Dab2 depletion reduced the lifetime of CCSs on collagen fibers (Fig. 4E). Thus, CCSs on collagen fibers experience “frustrated” endocytosis because budding forces may be balanced by the Dab2- and β 1-integrin-dependent engagement of CCSs with fibers.

TCALs regulate cell adhesion to collagen fibers

Next, we investigated whether the tight interaction we uncovered between CCSs/TCALs and ECM could play a role in cell adhesion. As expected, adhesion of MDA-MB-231 cells to a collagen-coated

Fig. 2. Ultrastructural characterization of TCALs.

(A to I) Survey view of the cytoplasmic surface of the plasma membrane in unroofed control [(A) to (D) and (G)] or CHC-depleted [(E), (F), (H), and (I)] MDA-MB-231 cells plated for 15 min on a thin layer of collagen fibers polymerized on glass. For (B) to (F), use view glasses for 3D viewing of anaglyphs (left eye, red). In (E), (G), and (H), α -adaptin was immunolabeled with gold particles (yellow dots). Arrows indicate collagen fibers. Pictures in (G) to (I) show areas of the plasma membrane in contact with the flat, collagen-coated glass coverslips and depict flat clathrin-coated lattice (G) and characteristic patches of proteinaceous material [(H) and (I)] positive for α -adaptin (H) and thus corresponding to the coat of clathrin-adaptors. Scale bars, (A) 1 μ m, (B) to (H) 500 nm, and (I) 200 nm.



substratum drastically depended on $\beta 1$ -integrin because virtually no cells could adhere upon incubation with a $\beta 1$ -integrin blocking antibody (fig. S7A). We next measured cell adhesion at 15 min after plating in cells depleted of AP-2 subunits, CHC, or dynamin-2. Cell adhesion to collagen-coated plastic dishes was not significantly affected upon inhibition of either of these proteins (fig. S7BC). However, plastic represents a rigid environment on which cell contractility is high (12), and FAs were readily detectable 15 min after plating (fig. S7D). On the other hand, cells showed very few detectable FAs 15 min after plating onto a 5-kilopascals (kPa) collagen-coated gel (fig. S7D). We reasoned that rapid FA formation on the non-physiological hard substratum may hinder a potential role for CCSs in cell adhesion. Indeed, AP-2 was required for optimal cell adhesion on the soft substratum (Fig. 4F). However, neither CHC nor dynamin-2 depletion inhibited cell adhesion on soft collagen (Fig. 4F). Because AP-2-positive structures are still visible at the plasma membrane in the absence of CHC or dynamin-2 (fig. S2, D and E) (29, 30), our results indicate that AP-2-dependent protein clustering at the plasma membrane, but not endocytosis per se, is required for optimal adhesion. Along this line, AP-2-positive structures were still able to cluster $\beta 1$ -integrin on fibers in the absence of CHC (fig. S7E). We also assessed the role of the integrin-binding, phosphotyrosine binding domain (PTB)-containing clathrin adaptors Numb, ARH, and Dab2 in the adhesion assays. Only Dab2 was required for cell adhesion on the soft substratum (Fig. 4F and fig. S7, B and F). We next investigated the possibility that adhesive TCALs may allow the cell to “grab” collagen fibers. When cells are plated on a thin layer of collagen fibers, they face a mixed environment because the glass coverslip is rigid but the network of collagen fibers shows some degree of flexibility and can be rearranged by cells (fig. S7G). Inhibiting the formation of CCSs using AP-2-specific small interfering RNAs (siRNAs) impaired collagen fiber rearrangement (Fig. 4G). Dab-2 was similarly required for fiber rearrangement (Fig. 4G). However, we did not detect rearrangement defects with CHC- or dynamin-2-silenced cells, indicating that cells can grab collagen fibers in an endocytosis-independent manner (Fig. 4G). We obtained similar results with cells that underwent two rounds of CHC depletion (Materials and methods) (fig. S7H). In addition, expression of a wild-type Dab2 construct was able to correct the remodeling defect seen in Dab2-depleted cells, but a PTB-deleted mutant, unable to recruit $\beta 1$ -integrins to clathrin structures (27), was not (fig. S7I). Both constructs were correctly localized (fig. S7J), suggesting that integrin recruitment by Dab2 in CCSs is crucial to grab and move collagen fibers. Thus, CCSs regulate cell adhesion to collagen fibers, which depends on CCSs’ capacity to locally cluster integrins at the cell surface.

TCALs stabilize cellular protrusions

If TCALs are adhesive structures pinching and grabbing collagen fibers, they may help cells to

migrate in the 3D network. Cell velocity in the 3D environment was reduced in AP-2- and Dab2-silenced cells and to a lesser extent in CHC- and dynamin-2-depleted cells (Fig. 5A). Cells treated with AP-2 or Dab2 siRNAs had a tendency to form more protrusions per unit of time as compared with that in controls, although this was not sta-

tistically significant (fig. S8A). In addition, AP-2 and Dab2, but not CHC, were required for optimal elongation of cellular protrusions (Fig. 5B). Similar differences were observed when measuring only the leading protrusion of actively migrating cells (fig. S8B). Thus, CCSs are required for the extension of long protrusions in an

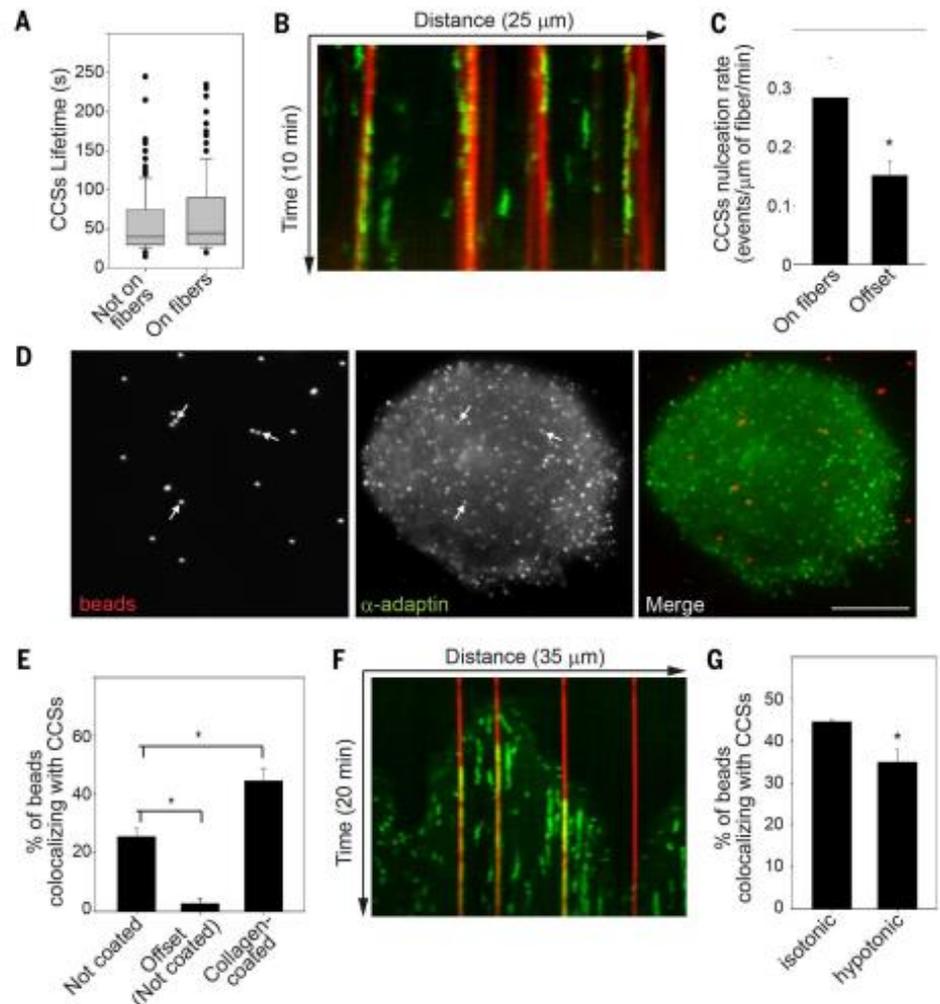


Fig. 3. Membrane curvature and collagen control TCAL accumulation on fibers. (A) Quantification of the lifetime of CCSs located on fibers or not, as indicated, in genome-edited MDA-MB-231 cells imaged for 10 min between 10 and 20 min after plating on a thin layer of collagen fibers polymerized on glass (mean \pm SEM; $n \geq 125$ CCSs, $*P < 0.01$, Student's *t* test). (B) Kymograph of a genome-edited MDA-MB-231 cell that was plated on a thin layer of collagen (red) fibers and imaged with spinning disk microscopy every 5 s for 10 min between 10 and 20 min after plating. CCSs are marked with the endogenous GFP-tagged $\mu 2$ -adaptin (green). (C) Quantification of CCS nucleation rate index on collagen fibers or after shifting the position of collagen fibers by 5 pixels to the right (Offset) (mean \pm SEM; $n = 3$ experiments, $*P < 0.01$, Student's *t* test). (D) MDA-MB-231 cells spreading on 200-nm-diameter beads (red) spotted on glass were fixed 15 min after plating and stained for α -adaptin (green). A merge picture is shown. Scale bar, 10 μ m. (E) Quantification of the percentage of uncoated or collagen-coated beads found below cells and colocalizing with CCSs. Positions of beads were shifted by 5 pixels to the right in the offset condition (mean \pm SEM; $n = 3$ experiments, $*P < 0.01$, Kruskal-Wallis one-way ANOVA). (F) Kymograph of a genome-edited MDA-MB-231 cell that was plated on 200-nm-diameter beads (red) spotted on a glass-bottom dish and imaged with spinning disk microscopy every 5 s for 20 min immediately after plating. CCSs are marked with the endogenous GFP-tagged $\mu 2$ -adaptin (green). (G) Quantification of the percentage of collagen-coated beads found below cells and colocalizing with CCSs 15 min after spreading in isotonic or hypotonic media as indicated (mean \pm SEM; $n = 3$ experiments, $*P < 0.01$, Student's *t* test).

endocytosis-independent manner and suggest that CCSs' roles in adhesion and endocytosis are two distinct functions that both participate in cell migration. Furthermore, CCS lifetime may not be a critical parameter in this process because CHC-depleted cells harbored very long-lived structures on collagen fibers [152.8 ± 43.8 s (SEM)] without any detectable consequence on protrusion dynamics and collagen remodeling capacity (Figs. 4G and 5B).

Our data suggest that TCALs may help the cell to migrate by anchoring protrusions to collagen fibers (movie S3). FAs are mostly observed at the extremities of elongated cells in the 3D network (fig. S8C) (13), whereas CCSs are distributed all over the cell's plasma membrane (Fig. 1A). We hypothesized that in addition to FAs, TCALs pinching collagen fibers may generate friction, helping the cell to build long protrusions and to migrate. To test this possibility, we laser ablated the FA-rich leading extremity of the main cell protrusion and monitored the consequences for its stability. Immediately after ablation, we observed a relaxation of the collagen network around the edge of the protrusion (Fig. 5C). Particle image velocimetry (PIV) analysis showed that relaxation occurred bidirectionally along the long axis of the protrusion

(Fig. 5C). Fiber relaxation away from the leading edge demonstrated that the cell exerts traction forces on the network. Relaxation in the direction of the cell body is most likely a consequence of protrusion retraction that occurred immediately after ablation of the extremity (Fig. 5, D and E). This suggested that the protrusion was under tension before ablation—most likely as a result of pulling forces exerted by the cell at FAs. Silencing AP-2 or Dab2, but not CHC, led to a more pronounced retraction of protrusions as compared with that in control cells (Fig. 5, D and E, and movie S4). Thus, CCSs stabilize cellular protrusions in an endocytosis-independent manner. Together, our data suggest that TCALs anchor the protrusion to collagen fibers and that this is critical to sustain high tension across the cell and to form long protrusions.

Outlook

Overall, we have found that TCALs are recruited at contact sites with collagen fibers and control cell adhesion to these fibers, powering cell migration in 3D environments. We propose that this mode of adhesion is critical for the mesenchymal mode of migration, in which TCALs provide several and dynamic anchoring points to collagen fibers, al-

lowing the cell to cope with FA-associated high tension across protrusions. Because ECM fibers are abundant in complex organisms, this mode of adhesion could play a general and universal role in helping cells to interact with their environment.

Materials and methods

Cell lines and constructs

MDA-MB-231 cells (a gift from P. Chavrier, Institut Curie, Paris, France) or genome edited MDA-MB-231 cells engineered to express an endogenous GFP-tagged $\mu 2$ subunit (a gift from D. Drubin, University of California-Berkeley, California, USA) were grown in DMEM Glutamax supplemented with 10% foetal calf serum at 37°C in 5% CO₂. DNA sequences encoding residues 1-766 (full length) or 172-766 (Δ PTB) of Dab2 were obtained by PCR by using cDNA of mouse Dab2 (a kind gift from M. Mettlen, University of Texas Southwestern Medical Center, Dallas, USA) as a template. PCR fragments with engineered flanking restriction sites were subcloned into the multi-cloning sites of pEGFP-C2 (Clontech) to encode in-frame fusion proteins with the amino-terminal EGFP-tag. MDA-MB-231 lines stably expressing EGFP, EGFP-Dab2 or EGFP-Dab2 Δ PTB were generated upon

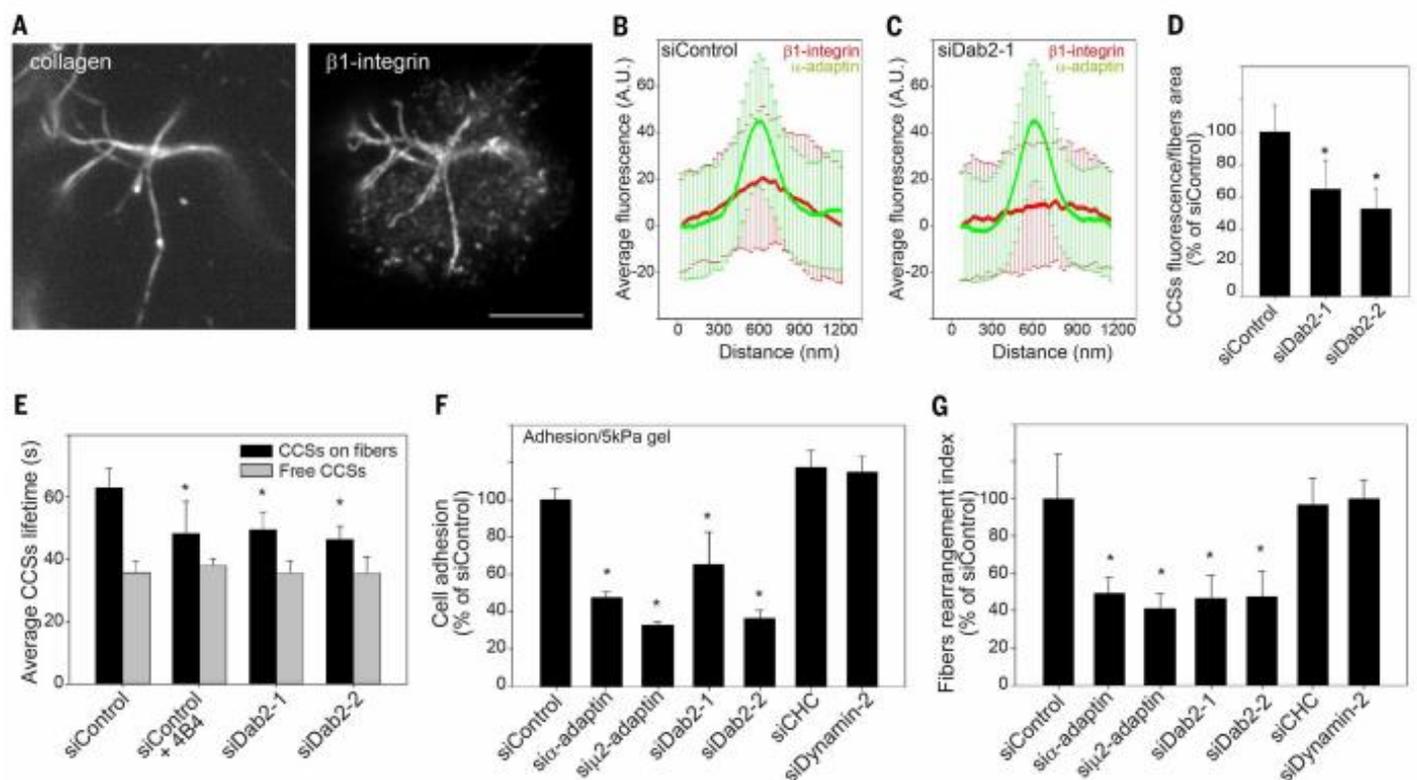


Fig. 4. TCALs regulate binding to collagen fibers. (A) MDA-MB-231 cells spreading on a layer of collagen fibers (left) were fixed and stained for activated $\beta 1$ -integrin (right) 15 min after plating. Scale bar, 10 μ m. (B and C) Average fluorescence distribution along collagen fibers of $\beta 1$ -integrin and α -adaptin in (B) control or (C) Dab2-depleted cells. Error bars indicate mean \pm SEM. (D) Average fluorescence intensity of α -adaptin staining per collagen fibers measured 15 min after plating in control or Dab2-depleted cells. (E) Average lifetime of CCSs in contact (black bars) or not (gray bars) with collagen fibers in the 3D network in cells treated with indicated

siRNAs and with or without 4B4 blocking antibody, as indicated. (F) Calcein-loaded MDA-MB-231 cells treated with the indicated siRNAs were plated on collagen-coated 5-kPa acrylamide gel for 15 min before washing unattached cells. Cell-associated fluorescence was measured and expressed as a percentage \pm SEM of control cells. (G) Quantification of collagen fibers movement measured during the first 15 min of spreading of MDA-MB-231 cells treated with the indicated siRNAs. For (D) to (G), data are expressed as mean \pm SEM; $n = 3$ experiments, * $P < 0.01$, Kruskal-Wallis one-way ANOVA.

transfection of cells with corresponding plasmids followed by selection using G418. These MDA-MB-231 lines were used for rescue experiments in the collagen fibers remodelling assay (see below) upon depletion of endogenous Dab2.

Antibodies and drugs

Mouse monoclonal anti-clathrin heavy chain (CHC) antibody and mouse monoclonal anti- α -adaptin antibody were obtained from BD Transduction Laboratories (Becton Dickinson France SAS, Le Pont-De-Claix, France). Rabbit polyclonal anti- α -adaptin antibodies (M300) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cortactin was obtained from Millipore and Vinculin antibody was a kind gift from Dr. M. Glukhova. HRP-conjugated anti-mouse and anti-rabbit antibodies for Western blot and Cy3-conjugated anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Alexa488-conjugated anti-mouse antibodies and Alexa545-labeled phalloidin were from Molecular Probes (Invitrogen). 4B4 antibody was obtained from Beckman coulter. Blebbistatin was purchased from Sigma and used a final concentration of 10 μ M.

RNA interference

For siRNA depletion, MDA-MB-231 cells were plated at 50% confluence and treated with the indicated siRNA (30 nM) using RNAiMax (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Protein depletion was maximal after 72 hours of siRNA treatment as shown by immunoblotting analysis with specific antibodies. For some experiments a modified protocol was used (see below). Equal loading of the cell lysates was verified by immunoblotting with anti-tubulin antibodies. The following siRNAs were used: α -adaptin, 5'-AUGGCGGUGGUGCGGCUCCTT-3'; μ 2-adaptin, 5'-AAGUGGAUGCCUUUCGGGUCA-3'; Clathrin heavy chain (CHC), 5'CGUGGGAAAACUCUUCAGATT-3'; Dynamin-2 SMARTpool siRNAs (Dharmacon); Talin, 5'-ACAAGAUGGAGAAUCAUUUU-3'; Dab2-1, 5'-GGUUGCCU-UAGUAGUCAATT-3'; Dab2-2, 5'-GAGCAUGAA-CAUCCAGAUAAATT-3'; ARH-1, 5'-GAUACAGCUU-GGCACUUUATT-3'; ARH-2, 5'-CAGACAAGAU-GACGACAATT-3'; Numb-1, 5'-GGACCUCUAGU-UGACCAGTT-3'; Numb-2, 5'-GAUAGUCGUUGGU-UCAUCATT-3'; non-targeting siRNAs (siControl), ON-TARGETplus Non-Targeting SMARTpool siRNAs (Dharmacon).

Fluorescent beads

Red fluorescent (580/605) 200 nm diameter carboxylate-modified fluospheres (ThermoFisher) were incubated or not overnight with 50 μ g/ml collagen in 0.02M acetic acid before being sonicated and spotted on collagen-coated glass coverslips or glass-bottom fluidishes (World Precision Instruments) for 30 min at 37°C. 300,000 parental or genome edited MDA-MB-231 cells were seeded in complete medium in 6 well plates or fluidishes and imaged immediately by spinning disk microscopy (see below) or fixed in ice-cold methanol at indicated time after plating and processed for immunofluorescence using anti- α -adaptin antibodies.

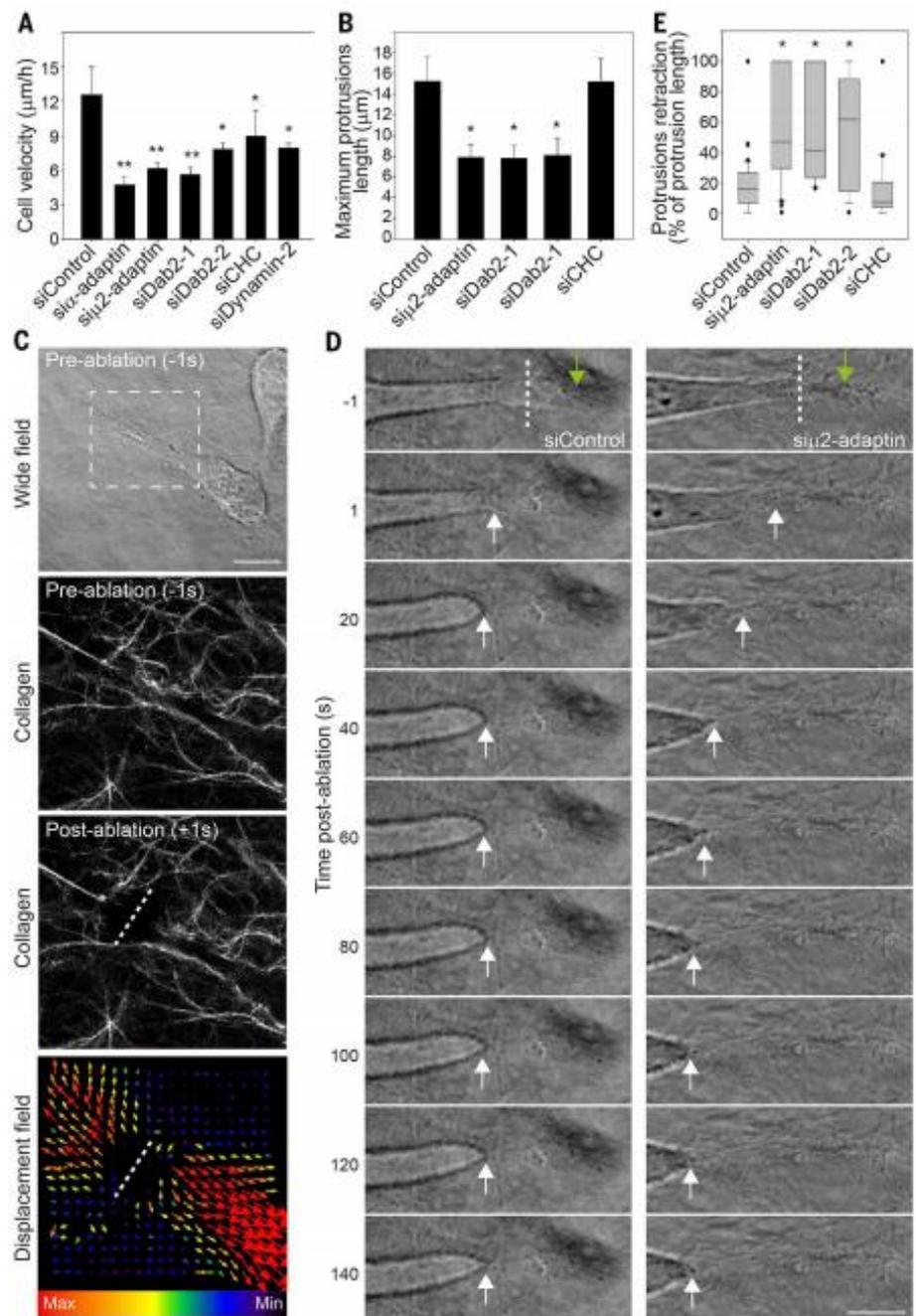


Fig. 5. TCALs regulate migration by stabilizing cell protrusions. (A) Average velocity of MDA-MB-231 cells treated with the indicated siRNA and migrating in the 3D collagen network (mean \pm SEM; $n = 3$ experiments, $*P < 0.05$, $**P < 0.01$, Kruskal-Wallis one-way ANOVA). (B) Average maximum elongation of all visible protrusions in MDA-MB-231 cells treated with the indicated siRNA and migrating in the 3D collagen network (mean \pm SEM; $n = 3$ experiments, $*P < 0.01$, Kruskal-Wallis one-way ANOVA). (C) The extremity of a MDA-MB-231 cell migrating in a 3D collagen network (top) was laser ablated. (Top middle and bottom middle) Collagen fibers corresponding to boxed area at top, 1 s before and after laser ablation, as indicated. The dashed line shows where ablation was performed. (Bottom) The calculated displacement field based on collagen fiber pictures 1 s before and after laser ablation, to illustrate collagen fiber movements upon relaxation. Color-coded bar of displacement intensity is shown. Scale bar, 10 μ m. (D) Representative galleries of protrusion retraction over 140 s after laser ablation of the extremity of MDA-MB-231 cells treated with the indicated siRNAs. Green arrows show the extremity of protrusions. Dashed lines show where laser ablation was performed. White arrows show the free extremity of retracting protrusions after ablation. Scale bar, 10 μ m. (E) Average distance of protrusion retraction over 3 min after laser ablation of their extremity in MDA-MB-231 cells treated with the indicated siRNA (mean \pm SEM; $n = 3$ experiments, $*P < 0.01$, Kruskal-Wallis one-way ANOVA).

At least 20 cells per conditions and per experiments in 3 independent experiments were analyzed.

Collagen networks

For preparation of a layer of thin collagen fibers, a 30 μ l droplet containing a 10:1 ratio of unlabeled and Alexa548-labeled collagen type I (rat tail acid extracted, BD bioscience) respectively, at a final concentration of 2.2 mg/ml was polymerized on glass coverslips or on glass-bottom dishes (Fluorodish, World Precision Instruments) at room temperature for 3 min before the excess of non-polymerized collagen being gently washed with PBS. The preparation was used immediately and immersed in complete medium seeded with 100,000 cells/ml. Cells were imaged during the spreading period by spinning disk microscopy (see below) or fixed with ice-cold methanol at the indicated time after plating and stained with indicated antibodies. For detection of β 1-integrin, cells were incubated at 4°C for 1 hour in the presence of the anti-activated β 1-integrin antibody (clone TS2/16, Santa Cruz) before to be fixed and processed for secondary and other antibodies labeling. At least 20 cells per conditions and per experiments in 3 independent experiments were analyzed.

For 3D cell migration assays, a 50 μ l droplet of collagen I at a final concentration of 2.2 mg/ml and seeded with 10,000 MDA-MB-231 cells was polymerized on glass-bottom dishes at room temperature for 30 min before to immerse the setup in pre-warmed complete medium. The next day, cells were imaged by phase contrast microscopy at 100 ms exposure every 20 min for 30 hours for migration assays or every 2 min for 4 hours for protrusion dynamics measurements. For CCSs dynamics analysis in the 3D environment, the droplet of collagen contained a 10:1 ratio of unlabeled and Alexa548-labeled collagen, respectively, and cells were imaged the next day by spinning disk microscopy (see below). In all cases, a single optical section was selected and imaged. Manual tracking of cell migration and measurement of protrusion or CCSs dynamics parameters were performed using Metamorph software. At least 10 cells per conditions and per experiments from 3 independent experiments were analyzed.

Indirect immunofluorescence microscopy and fluorescence quantification

MDA-MB-231 cells plated on the top of a thin layer of collagen fibers on coverslips or on the top of fluorescent beads spotted on collagen-coated coverslips were fixed in ice-cold methanol and processed for immunofluorescence microscopy by using the indicated antibodies. Cells were imaged through a 100 \times 1.40NA UPlanSApo objective lens of a wide-field IX73 microscope (Olympus) equipped with an Orca-Flash2.8 CMOS camera (Hamamatsu) and steered by CellSens Dimension software (Olympus).

For anti-vinculin and β 1-integrin staining, cells were briefly extracted for 1 min using 0.1% Triton prior to fixation.

For calculating the degree of CCSs alignment along collagen fibers, collagen fibers were segmented using Fiji software and the average fluo-

rescence intensity of the anti- α -adaplin staining in fibers area was measured for each individual cells and normalized to the area occupied by collagen fibers. At least 20 cells per conditions and per experiments in 3 independent experiments were analyzed. Data are expressed as a mean percentage of control situation \pm SEM.

For 3D immunofluorescence analyses, cells were fixed with 4% PFA 24 hours after embedding in the collagen network containing a 10:1 ratio of unlabeled and Alexa548-labeled collagen. Cells were stained using anti- α -adaplin antibodies, (AP6 clone, ThermoFisher). Stacks of images were collected along the z-axis with a 0.2 μ m interval between optical sections and cells were reconstructed in 3D using Imaris software. Distance between each individual α -adaplin-positive dots and the most adjacent collagen fibers were automatically measured upon segmentation of both structures using the Spots-Close-To-Surface XTension tool of Imaris software. Parameters used to segment objects were: 0.8 μ m XY and 1.5 μ m Z diameters for spot detection in the green channel (AP-2 staining) and 0.8 μ m spherical diameter for the Surfaces objects detection module in the red channel (collagen fibers). A distance of less than 0.5 μ m was considered a positive hit (see movie S1). To assess the specificity of colocalization, the same process was applied to the same cells matched with irrelevant collagen network regions. 12 cells per experiments in 2 independent experiments were analyzed.

Electron microscopy of unroofed cells

Adherent PM from MDA-MB-231 cells plated for 15 or 30 min on glass coverslips coated with a thin layer of collagen were disrupted by sonication as described previously (31). Glutaraldehyde/paraformaldehyde-fixed cells were further sequentially treated with OsO₄, tannic acid and uranyl acetate prior to dehydration and Hexamethyldisilazane drying (HMDS, Sigma-Aldrich). Dried samples were then rotary-shadowed with platinum and carbon with a high vacuum sputter coater (Leica). Platinum replicas were floated off the glass by angled immersion into hydrofluoric acid, washed several times by floatation on distilled water, and picked up on 200 mesh formvar/carbon-coated EM grids. The grids were mounted in a eucentric side-entry goniometer stage of a transmission electron microscope operated at 80 kV (Philips, model CM120) and images were recorded with a Morada digital camera (Olympus). Images were processed in Adobe Photoshop to adjust brightness and contrast and presented in inverted contrast. Anaglyphs were made by converting the -10° tilt image to red and the +10° tilt image to cyan (blue/green), layering them on top of each other using the screen blending mode in Adobe Photoshop, and aligning them to each other.

For analyzes of CHC-depleted cells, cells were transfected once with CHC-specific siRNAs and then a second time, 48 hours later, with the same siRNAs to completely eliminate any trace of clathrin at the plasma membrane of most cells as visible on EM metal replica. Cells were analyzed 96 hours after the first transfection. Of note, this protocol did not produce different functional results as compared to

a single round of siRNA transfection as confirmed using the collagen remodeling assay (fig. S8G).

Electron microscopy of cells in 3D

For electron microscopy of MDA-MB-231 cells cultured in a 3D network of collagen fibers, cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and then washed with PBS and incubated for 1 hour with 1% osmium tetroxide. The samples were then dehydrated and processed for Epon resin embedding and ultrathin sections and then contrasted with uranyl acetate and lead citrate. Samples were observed under an electron microscope (Philips CM120; FEI Company, Eindhoven, The Netherlands) and digital acquisitions were made with a numeric camera (Keen View; Soft Imaging System, Germany).

Spinning disk microscopy

Genome edited MDA-MB-231 cells were imaged for exposure times of 200 ms 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 60 \times 1.35NA UPLSAPO objective lens and a laser combiner system, which included 491 and 561 nm 100 mW DPSS lasers (Andor). Images were acquired with a Zyla sCMOS camera (Andor). The system was steered by IQ3 software (Andor). For genome edited MDA-MB-231 cells cultured in 3D collagen network, a z-stack of 3-4 frames at 0.25 μ m interval were acquired every 5 s. Subsequent quantifications were performed only on one selected optical section.

For calculating the nucleation index of CCSs on collagen fibers, collagen fibers were segmented using Metamorph software. 2-3 unambiguously individual fibers were selected per cell and the number of μ 2-adaplin-GFP spots appearing along these fibers in a 10 min time window was measured. Nucleation index was calculated by dividing this number by time (in min) and by the length of the collagen fibers (in μ m). As a control, the same analysis was performed after shifting the position of the fiber by approximately 10 pixels so that the virtual fiber remained in the cell and did not overlap with an actual fiber. 15 cells from 2 independent experiments were analyzed. Data are expressed as a mean percentage of control situation \pm SEM.

For measuring the lifetime of CCSs in 2D or 3D conditions, time periods between first and last detection of individual μ 2-adaplin-GFP spots on fibers or in other area of the cell were measured manually using Metamorph software in 2 different 60 \times 60 pixels large regions per cell over a 5 min period. At least 8 cells from 2 independent experiments were analyzed for a total of at least 125 CCSs per conditions.

For measuring the movement of collagen fibers, MDA-MB-231 spreading on the top of a thin layer of collagen fibers were imaged every 2 min for 30 min by spinning disk microscopy. To calculate the movement index, fibers were segmented using Metamorph software and the cumulative area occupied by fibers below individual cells during the 30 min acquisition window was measured and divided by the area occupied by the

same fibers in the first frame of the movie. At least 30 cells per conditions from 3 independent experiments were analyzed. Data are expressed as a mean percentage of control situation \pm SEM.

TIRF microscopy

For total internal reflection fluorescent microscopy (TIRF), MDA-MB-231 cells seeded onto collagen-fibers-coated glass coverslips were fixed in ice-cold methanol and stained with indicated antibodies before being imaged through a 100x 1.49 NA TIRF objective lens on a Nikon TE2000 (Nikon France SAS, Champigny sur Marne, France) inverted microscope equipped with a QuantEM EMCCD camera (Roper Scientific SAS, Evry, France / Photometrics, AZ, USA), a dual output laser launch, which included 491 and 561 nm 50 mW DPSS lasers (Roper Scientific), 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.

Laser ablation

Laser ablation was performed on a Leica Sp8 confocal microscope equipped with a Pecon incubation chamber to maintain the cells at 37°C and 5% CO₂. The FRAP wizard of the Leica software was used to laser ablate cell protrusions. Briefly, wide-field images of MDA-MB-231 cells migrating in the 3D collagen network were acquired 1s before laser ablation. Laser ablation was then performed in two-photon mode with 2 consecutive 1s laser pulses at 820 nm and 100% of power focused on the specimen through a 63x 1.4 NA objective. Cells were imaged immediately after ablation every 1s for 3 min. Displacement field of collagen fibers was calculated using the PIV plugin running under ImageJ (32) based on fluorescence pictures of Cy-3-labeled collagen fibers acquired 1s before and 1s after laser ablation.

Adhesion assays

MDA-MB-231 cells treated with indicated siRNAs were incubated with 5 μ M calcein (Sigma) for 30 min in DMEM to label cells. Cells were then washed in PBS, harvested using Versene (PBS, 0.02% EDTA) for 5 min at 37°C and resuspended in DMEM 10% FCS. 24 wells polystyrene plates (Falcon) or 5 kPa 24 wells soft plates (ExCellence) were incubated for 60 min at 37°C with 50 μ g/ml collagen in 0.02M acetic acid. 400,000 cells were seeded per well and allowed to adhere on the collagen-coated polystyrene or soft plates for 15 min at 37°C. Cell-associated calcein fluorescence was then immediately measured using a FLUOstar Optima plate reader (BMG Labtech) before and after washing thoroughly wells 3 times with PBS. Ratio between fluorescence levels before and after washing was used to calculate the adhesion index. Experiments were performed 3-5 times in triplicate. Results are expressed as a mean percentage of control cells (siControl) \pm SEM.

Statistical analyses

Statistical analyses in Figs. 3E; D to G; and 5, A, B, and E; and figs. S2I; S7, C, F, and I; and S8, A

and B have been performed using Kruskal-Wallis One Way Analysis of Variance (ANOVA) followed by an All Pairwise Multiple Comparison Procedure (Tukey Test). Data in Figs. 1, D and G; and 3, C and G; and figs. S1D and S7, A and H, have been tested using Student's *t* test. Statistical analysis of Imaris-extracted data on the percentage of CCSs contacting collagen fibers in the 3D environment was performed using Student's *t* test. All statistical analyses were performed using SigmaStat software.

REFERENCES AND NOTES

1. G. Montagnac, A. Echard, P. Chavrier, Endocytic traffic in animal cell cytokinesis. *Curr. Opin. Cell Biol.* 20, 454–461 (2008). doi: 10.1016/j.cob.2008.03.011; pmid: 18472411
2. T. Maritzen, H. Schachtner, D. F. Legler, On the move: Endocytic trafficking in cell migration. *Cell. Mol. Life Sci.* 72, 2119–2134 (2015). doi: 10.1007/s00018-015-1855-9; pmid: 25681867
3. H. T. McMahon, E. Boucrot, Molecular mechanism and physiological functions of clathrin-mediated endocytosis. *Nat. Rev. Mol. Cell Biol.* 12, 517–533 (2011). doi: 10.1038/nrm3151; pmid: 21779028
4. N. R. Paul, G. Jacquemet, P. T. Caswell, Endocytic trafficking of integrins in cell migration. *Curr. Biol.* 25, R1092–R1105 (2015). doi: 10.1016/j.cub.2015.09.049; pmid: 26583903
5. E. J. Ezratty, C. Bertaux, E. E. Marcantonio, G. G. Gundersen, Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. *J. Cell Biol.* 187, 733–747 (2009). doi: 10.1083/jcb.200904054; pmid: 19951918
6. W. T. Chao, J. Kunz, Focal adhesion disassembly requires clathrin-dependent endocytosis of integrins. *FEBS Lett.* 583, 1337–1343 (2009). doi: 10.1016/j.febslet.2009.03.037; pmid: 19306879
7. E. M. Balchelder, D. Yasar, Differential requirements for clathrin-dependent endocytosis at sites of cell-substrate adhesion. *Mol. Biol. Cell* 21, 3070–3079 (2010). doi: 10.1091/mbc.E09-12-1044; pmid: 20631253
8. A. P. Liu, D. Loefer, S. L. Schmid, G. Danuser, Global and local regulation of clathrin-coated pit dynamics detected on patterned substrates. *Biophys. J.* 97, 1038–1047 (2009). doi: 10.1016/j.bpj.2009.06.003; pmid: 19686651
9. N. Tawil, P. Wilson, S. Carbonetto, Integrins in point contacts mediate cell spreading: Factors that regulate integrin accumulation in point contacts vs. focal contacts. *J. Cell Biol.* 120, 261–271 (1993). doi: 10.1083/jcb.120.1.261; pmid: 8416993
10. P. Maupin, T. D. Pollard, Improved preservation and staining of HeLa cell actin filaments, clathrin-coated membranes, and other cytoplasmic structures by tannic acid-glutaraldehyde-saponin fixation. *J. Cell Biol.* 96, 51–62 (1983). doi: 10.1083/jcb.96.1.51; pmid: 6186673
11. M. Lampe, S. Vassilopoulos, C. Merrifield, Clathrin coated pits, plaques and adhesion. *J. Struct. Biol.* 196, 48–56 (2016). doi: 10.1016/j.jsb.2016.07.009; pmid: 27431447
12. D. E. Discher, P. Janmey, Y. L. Wang, Tissue cells feel and respond to the stiffness of their substrate. *Science* 310, 1139–1143 (2005). doi: 10.1126/science.1116995; pmid: 16293750
13. S. Geraldo et al., Do cancer cells have distinct adhesions in 3D collagen matrices and in vivo? *Eur. J. Cell Biol.* 91, 930–937 (2012). doi: 10.1016/j.ejcb.2012.07.005; pmid: 22939225
14. J. T. Parsons, A. R. Horwitz, M. A. Schwartz, Cell adhesion: Integrating cytoskeletal dynamics and cellular tension. *Nat. Rev. Mol. Cell Biol.* 11, 633–643 (2010). doi: 10.1038/nrm2957; pmid: 20729930
15. R. J. Pelham Jr., Y.-I. Wang, Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc. Natl. Acad. Sci. U.S.A.* 94, 13661–13665 (1997). doi: 10.1073/pnas.94.25.13661; pmid: 9391082
16. E. Cukierman, R. Pankov, D. R. Stevens, K. M. Yamada, Taking cell-matrix adhesions to the third dimension. *Science* 294, 1708–1712 (2001). doi: 10.1126/science.1064829; pmid: 11721053
17. G. Montagnac et al., α TAT1 catalyses microtubule acetylation at clathrin-coated pits. *Nature* 502, 567–570 (2013). doi: 10.1038/nature12571; pmid: 24097348
18. S. H. Hong, C. L. Cortesio, D. G. Drubin, Machine-learning-based analysis in genome-edited cells reveals the efficiency of clathrin-mediated endocytosis. *Cell Rep.* 12, 2121–2130 (2015). doi: 10.1016/j.celrep.2015.08.048; pmid: 26387943

19. H. Yamaguchi, J. Wycloff, J. Condeelis, Cell migration in tumors. *Curr. Opin. Cell Biol.* 17, 559–564 (2005). doi: 10.1016/j.cob.2005.08.002; pmid: 16098726
20. P. Monteiro et al., Endosomal WASH and exocyst complexes control exocytosis of MT1-MMP at invadopodia. *J. Cell Biol.* 203, 1063–1079 (2013). doi: 10.1083/jcb.201306162; pmid: 24344185
21. N. C. Gauthier, M. A. Fardin, P. Roca-Cusachs, M. P. Sheetz, Temporary increase in plasma membrane tension coordinates the activation of exocytosis and contraction during cell spreading. *Proc. Natl. Acad. Sci. U.S.A.* 108, 14467–14472 (2011). doi: 10.1073/pnas.1105845108; pmid: 21808040
22. A. Pietuch, A. Janshoff, Mechanics of spreading cells probed by atomic force microscopy. *Open Biol.* 3, 130084 (2013). doi: 10.1098/rsob.130084; pmid: 23864554
23. K. Keren, Cell motility: The integrating role of the plasma membrane. *Eur. Biophys. J.* 40, 1013–1027 (2011). doi: 10.1007/s00249-011-0741-0; pmid: 21833780
24. T. J. Pucadyil, S. S. Holkar, Comparative analysis of adaptor-mediated clathrin assembly reveals general principles for adaptor clustering. *Mol. Biol. Cell* 27, 3156–3163 (2016). doi: 10.1091/mbc.E16-06-0399; pmid: 27559129
25. A. D. Doyle, N. Carvajal, A. Jin, K. Matsumoto, K. M. Yamada, Local 3D matrix microenvironment regulates cell migration through spatiotemporal dynamics of contractility-dependent adhesions. *Nat. Commun.* 6, 8720 (2015). doi: 10.1038/ncomms9720; pmid: 26548801
26. A. Teekchandani et al., Quantitative proteomics identifies a Dab2/integrin module regulating cell migration. *J. Cell Biol.* 186, 99–111 (2009). doi: 10.1083/jcb.200812160; pmid: 19581412
27. D. Chetrit, N. Ziv, M. Ehrlich, Dab2 regulates clathrin assembly and cell spreading. *Biochem. J.* 418, 701–715 (2009). doi: 10.1042/BJ20081288; pmid: 19000037
28. C. Prunier, P. H. Howe, Disabled-2 (Dab2) is required for transforming growth factor beta-induced epithelial to mesenchymal transition (EMT). *J. Biol. Chem.* 280, 17540–17548 (2005). doi: 10.1074/jbc.M500974200; pmid: 15734730
29. A. Motley, N. A. Bright, M. N. Seaman, M. S. Robinson, Clathrin-mediated endocytosis in AP-2-depleted cells. *J. Cell Biol.* 162, 909–918 (2003). doi: 10.1083/jcb.200305145; pmid: 12952941
30. L. Hinrichsen, J. Harborth, L. Andrees, K. Weber, E. J. Ungewickell, Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. *J. Biol. Chem.* 278, 45160–45170 (2003). doi: 10.1074/jbc.M307290200; pmid: 12960147
31. J. Heuser, The production of 'cell cortices' for light and electron microscopy. *Traffic* 1, 545–552 (2000). doi: 10.1034/j.1600-0854.2000.010704.x; pmid: 11208142
32. Q. Tseng et al., Spatial organization of the extracellular matrix regulates cell-cell junction positioning. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1506–1511 (2012). doi: 10.1073/pnas.1106377109; pmid: 22307605

ACKNOWLEDGMENTS

The authors thank M. Piet, B. Ladoux, P. Chavrier, and J. Ivaska for critical comments on the manuscript. We thank the Gustave Roussy imaging facility for help with image acquisition and the Electron Microscopy Facility of the Institut de Biologie Paris-Seine (IBPS). Core funding for this work was provided by the Gustave Roussy Institute and INSERM, and additional support was provided by grants from ATP/Avenir Program and la Ligue Nationale Contre le Cancer and from the Agence Nationale de la Recherche (ANR-15-CE15-0005-03) to G.M. and a young researcher grant (ANR-14-CE12-0001-01) to S.V. N.E. designed and performed experiments, analyzed results, and wrote the manuscript. E.B., F.B., and A.L.R. performed experiments. G.V.N. designed and performed electron microscopy analyses in the 3D environment. S.V. designed and performed metal-replica electron microscopy analyses and wrote the manuscript. G.M. supervised the study, designed experiments, and wrote the manuscript. Data described can be found in the main figures and supplementary materials. The authors declare no competing financial interests.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/356/6343/eaal4713/suppl/DC1
Figs. S1 to S8
Movies S1 to S4

24 November 2016; resubmitted 10 April 2017
Accepted 11 May 2017
10.1126/science.aal4713

REFERENCES

- Aguet, François et al. 2013. 'Advances in Analysis of Low Signal-to-Noise Images Link Dynamin and AP2 to the Functions of an Endocytic Checkpoint'. *Developmental Cell* 26(3): 279–91.
- Ahuja, Rashmi et al. 2007. 'Cordon-Bleu Is an Actin Nucleation Factor and Controls Neuronal Morphology'. *Cell* 131(2): 337–50.
- Albuschies, Jörg, and Viola Vogel. 2013. 'The Role of Filopodia in the Recognition of Nanotopographies'. *Scientific Reports* 3: 1658.
- Alexandrova, Antonina Y. et al. 2008. 'Comparative Dynamics of Retrograde Actin Flow and Focal Adhesions: Formation of Nascent Adhesions Triggers Transition from Fast to Slow Flow'. *PLOS ONE* 3(9): e3234.
- Alon, R. et al. 1994. 'TNF-Alpha Binds to the N-Terminal Domain of Fibronectin and Augments the Beta 1-Integrin-Mediated Adhesion of CD4+ T Lymphocytes to the Glycoprotein'. *Journal of Immunology (Baltimore, Md.: 1950)* 152(3): 1304–13.
- Aman, Andy, and Tatjana Piotrowski. 2010. 'Cell Migration during Morphogenesis'. *Developmental Biology* 341(1): 20–33.
- An, Bo, Yu-Shan Lin, and Barbara Brodsky. 2016. 'Collagen Interactions: Drug Design and Delivery.' *Advanced drug delivery reviews* 97: 69–84.
- Anderson, R. G., M. S. Brown, and J. L. Goldstein. 1977. 'Role of the Coated Endocytic Vesicle in the Uptake of Receptor-Bound Low Density Lipoprotein in Human Fibroblasts'. *Cell* 10(3): 351–64.
- Andrew, Natalie, and Robert H. Insall. 2007. 'Chemotaxis in Shallow Gradients Is Mediated Independently of PtdIns 3-Kinase by Biased Choices between Random Protrusions'. *Nature Cell Biology* 9(2): 193–200.
- Andrianantoandro, Ernesto, and Thomas D. Pollard. 2006. 'Mechanism of Actin Filament Turnover by Severing and Nucleation at Different Concentrations of ADF/Cofilin'. *Molecular Cell* 24(1): 13–23.
- Antonescu, Costin N., François Aguet, Gaudenz Danuser, and Sandra L. Schmid. 2011. 'Phosphatidylinositol-(4,5)-Bisphosphate Regulates Clathrin-Coated Pit Initiation, Stabilization, and Size'. *Molecular Biology of the Cell* 22(14): 2588–2600.
- Antonny, Bruno et al. 2016. 'Membrane Fission by Dynamin: What We Know and What We Need to Know'. *The EMBO journal* 35(21): 2270–84.
- Appert-Collin, Aline, Pierre Hubert, Gérard Crémel, and Amar Bennisroune. 2015. 'Role of ErbB Receptors in Cancer Cell Migration and Invasion'. *Frontiers in Pharmacology* 6. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4657385/> (October 16, 2019).

- Arjonen, Antti et al. 2014. 'Mutant P53-Associated Myosin-X Upregulation Promotes Breast Cancer Invasion and Metastasis'. *The Journal of Clinical Investigation* 124(3): 1069–82.
- Arriemerlou, Cécile, and Tobias Meyer. 2005. 'A Local Coupling Model and Compass Parameter for Eukaryotic Chemotaxis'. *Developmental Cell* 8(2): 215–27.
- Attieh, Youmna et al. 2017. 'Cancer-Associated Fibroblasts Lead Tumor Invasion through Integrin-B3-Dependent Fibronectin Assembly'. *The Journal of Cell Biology* 216(11): 3509–20.
- Bailly, M. et al. 1998. 'Regulation of Protrusion Shape and Adhesion to the Substratum during Chemotactic Responses of Mammalian Carcinoma Cells'. *Experimental Cell Research* 241(2): 285–99.
- Bailly, Maryse et al. 2000. 'Epidermal Growth Factor Receptor Distribution during Chemotactic Responses'. *Molecular Biology of the Cell* 11(11): 3873–83.
- Balaban, N. Q. et al. 2001. 'Force and Focal Adhesion Assembly: A Close Relationship Studied Using Elastic Micropatterned Substrates'. *Nature Cell Biology* 3(5): 466–72.
- Ballestrem, Christoph et al. 2006. 'Molecular Mapping of Tyrosine-Phosphorylated Proteins in Focal Adhesions Using Fluorescence Resonance Energy Transfer'. *Journal of Cell Science* 119(Pt 5): 866–75.
- Barczyk, Malgorzata, Sergio Carracedo, and Donald Gullberg. 2010. 'Integrins'. *Cell and Tissue Research* 339(1): 269–80.
- Barriga, Elias H., and Roberto Mayor. 2015. 'Embryonic Cell-Cell Adhesion: A Key Player in Collective Neural Crest Migration'. *Current Topics in Developmental Biology* 112: 301–23.
- Baruzzi, A., E. Cavegion, and G. Berton. 2008. 'Regulation of Phagocyte Migration and Recruitment by Src-Family Kinases'. *Cellular and molecular life sciences: CMLS* 65(14): 2175–90.
- Baschieri, Francesco et al. 2018. 'Frustrated Endocytosis Controls Contractility-Independent Mechanotransduction at Clathrin-Coated Structures'. *Nature Communications* 9(1): 1–13.
- Bashkirov, Pavel V. et al. 2008. 'GTPase Cycle of Dynamin Is Coupled to Membrane Squeeze and Release, Leading to Spontaneous Fission'. *Cell* 135(7): 1276–86.
- Batchelder, Erika M., and Defne Yarar. 2010. 'Differential Requirements for Clathrin-Dependent Endocytosis at Sites of Cell-Substrate Adhesion'. *Molecular Biology of the Cell* 21(17): 3070–79.
- Baulida, J. et al. 1996. 'All ErbB Receptors Other than the Epidermal Growth Factor Receptor Are Endocytosis Impaired'. *The Journal of Biological Chemistry* 271(9): 5251–57.
- Bearer, E. L. 1991. 'Direct Observation of Actin Filament Severing by Gelsolin and Binding by GCap39 and CapZ'. *The Journal of Cell Biology* 115(6): 1629–38.
- Beglova, Natalia, and Stephen C. Blacklow. 2005. 'The LDL Receptor: How Acid Pulls the Trigger'. *Trends in Biochemical Sciences* 30(6): 309–17.
- Benesch, Stefanie et al. 2005. 'N-WASP Deficiency Impairs EGF Internalization and Actin Assembly at Clathrin-Coated Pits'. *Journal of Cell Science* 118(14): 3103–15.

- Beningo, K. A. et al. 2001. 'Nascent Focal Adhesions Are Responsible for the Generation of Strong Propulsive Forces in Migrating Fibroblasts'. *The Journal of Cell Biology* 153(4): 881–88.
- Benmerah, Alexandre, and Christophe Lamaze. 2007. 'Clathrin-Coated Pits: Vive La Différence?' *Traffic* 8(8): 970–82.
- Bhattacharjee, Arnab, and Manju Bansal. 2005. 'Collagen Structure: The Madras Triple Helix and the Current Scenario'. *IUBMB life* 57(3): 161–72.
- Björkelund, Hanna, Lars Gedda, and Karl Andersson. 2011. 'Comparing the Epidermal Growth Factor Interaction with Four Different Cell Lines: Intriguing Effects Imply Strong Dependency of Cellular Context'. *PLoS One* 6(1): e16536.
- Blaser, Heiko et al. 2006. 'Migration of Zebrafish Primordial Germ Cells: A Role for Myosin Contraction and Cytoplasmic Flow'. *Developmental Cell* 11(5): 613–27.
- Boekhorst, Veronika Te, and Peter Friedl. 2016. 'Plasticity of Cancer Cell Invasion-Mechanisms and Implications for Therapy.' *Advances in cancer research* 132: 209–64.
- Bosman, Fred T., and Ivan Stamenkovic. 2003. 'Functional Structure and Composition of the Extracellular Matrix'. *The Journal of Pathology* 200(4): 423–28.
- Bouchard, Frédéric, Simon D. Bélanger, Katherine Biron-Pain, and Yves St-Pierre. 2010. 'EGR-1 Activation by EGF Inhibits MMP-9 Expression and Lymphoma Growth'. *Blood* 116(5): 759–66.
- Boucrot, Emmanuel et al. 2015. 'Endophilin Marks and Controls a Clathrin-Independent Endocytic Pathway'. *Nature* 517(7535): 460–65.
- Boulant, Steeve et al. 2011. 'Actin Dynamics Counteract Membrane Tension during Clathrin-Mediated Endocytosis'. *Nature Cell Biology* 13(9): 1124–31.
- Bovellan, Miia et al. 2014. 'Cellular Control of Cortical Actin Nucleation'. *Current biology: CB* 24(14): 1628–35.
- Bray, D., and J. G. White. 1988. 'Cortical Flow in Animal Cells'. *Science (New York, N.Y.)* 239(4842): 883–88.
- Bretscher, M S. 1989. 'Endocytosis and Recycling of the Fibronectin Receptor in CHO Cells.' *The EMBO Journal* 8(5): 1341–48.
- Bretscher, M S. 1992. 'Cells Can Use Their Transferrin Receptors for Locomotion.' *The EMBO Journal* 11(2): 383–89.
- Brown, Claire M. et al. 2006. 'Probing the Integrin-Actin Linkage Using High-Resolution Protein Velocity Mapping'. *Journal of Cell Science* 119(24): 5204–14.
- Brown, M. S., and J. L. Goldstein. 1979. 'Receptor-Mediated Endocytosis: Insights from the Lipoprotein Receptor System'. *Proceedings of the National Academy of Sciences of the United States of America* 76(7): 3330–37.
- Brundage, R. A., K. E. Fogarty, R. A. Tuft, and F. S. Fay. 1991. 'Calcium Gradients Underlying Polarization and Chemotaxis of Eosinophils'. *Science (New York, N.Y.)* 254(5032): 703–6.

- Buehler, Markus J. 2006. 'Nature Designs Tough Collagen: Explaining the Nanostructure of Collagen Fibrils'. *Proceedings of the National Academy of Sciences of the United States of America* 103(33): 12285–90.
- Burgess, Antony W et al. 2003. 'An Open-and-Shut Case? Recent Insights into the Activation of EGF/ErbB Receptors'. *Molecular Cell* 12(3): 541–52.
- Burnette, Dylan T. et al. 2011. 'A Role for Actin Arcs in the Leading-Edge Advance of Migrating Cells'. *Nature Cell Biology* 13(4): 371–81.
- Burridge, Keith et al. 1988. 'Focal Adhesions: Transmembrane Junctions Between the Extracellular Matrix and the Cytoskeleton'. *Annual Review of Cell Biology* 4(1): 487–525.
- Bustos, Rodrigo I., Marie-Annick Forget, Jeffrey E. Settleman, and Steen H. Hansen. 2008. 'Coordination of Rho and Rac GTPase Function via P190B RhoGAP'. *Current biology: CB* 18(20): 1606–11.
- Byyny, R. L., D. N. Orth, S. Cohen, and E. S. Doyne. 1974. 'Epidermal Growth Factor: Effects of Androgens and Adrenergic Agents'. *Endocrinology* 95(3): 776–82.
- Campellone, Kenneth G., and Matthew D. Welch. 2010. 'A Nucleator Arms Race: Cellular Control of Actin Assembly'. *Nature Reviews Molecular Cell Biology* 11(4): 237–51.
- Carpenter, G., and S. Cohen. 1979. 'Epidermal Growth Factor'. *Annual Review of Biochemistry* 48: 193–216.
- Carpentier, J. L., M. F. White, L. Orci, and R. C. Kahn. 1987. 'Direct Visualization of the Phosphorylated Epidermal Growth Factor Receptor during Its Internalization in A-431 Cells'. *The Journal of Cell Biology* 105(6 Pt 1): 2751–62.
- Carter, S. B. 1965. 'Principles of Cell Motility: The Direction of Cell Movement and Cancer Invasion'. *Nature* 208(5016): 1183–87.
- Caswell, Patrick T. et al. 2007. 'Rab25 Associates with Alpha5beta1 Integrin to Promote Invasive Migration in 3D Microenvironments'. *Developmental Cell* 13(4): 496–510.
- Caswell, Patrick T., and Jim C. Norman. 2006. 'Integrin Trafficking and the Control of Cell Migration'. *Traffic (Copenhagen, Denmark)* 7(1): 14–21.
- Chen, Zhucheng et al. 2010. 'Structure and Control of the Actin Regulatory WAVE Complex'. *Nature* 468(7323): 533–38.
- Chernousov, M. A., F. J. Fogerty, V. E. Kotliansky, and D. F. Mosher. 1991. 'Role of the I-9 and III-1 Modules of Fibronectin in Formation of an Extracellular Fibronectin Matrix'. *The Journal of Biological Chemistry* 266(17): 10851–58.
- Choi, Colin K. et al. 2008. 'Actin and Alpha-Actinin Orchestrate the Assembly and Maturation of Nascent Adhesions in a Myosin II Motor-Independent Manner'. *Nature Cell Biology* 10(9): 1039–50.
- Chrzanowska-Wodnicka, M., and K. Burridge. 1996. 'Rho-Stimulated Contractility Drives the Formation of Stress Fibers and Focal Adhesions'. *The Journal of Cell Biology* 133(6): 1403–15.

- Citri, Ami, and Yosef Yarden. 2006. 'EGF-ERBB Signalling: Towards the Systems Level'. *Nature Reviews. Molecular Cell Biology* 7(7): 505–16.
- Clayton, Andrew H. A. et al. 2005. 'Ligand-Induced Dimer-Tetramer Transition during the Activation of the Cell Surface Epidermal Growth Factor Receptor-A Multidimensional Microscopy Analysis'. *The Journal of Biological Chemistry* 280(34): 30392–99.
- Cocucci, Emanuele, François Aguet, Steeve Boulant, and Tom Kirchhausen. 2012. 'The First Five Seconds in the Life of a Clathrin-Coated Pit'. *Cell* 150(3): 495–507.
- Cohen, Stanley. 1960. 'PURIFICATION OF A NERVE-GROWTH PROMOTING PROTEIN FROM THE MOUSE SALIVARY GLAND AND ITS NEURO-CYTOTOXIC ANTISERUM*'. *Proceedings of the National Academy of Sciences of the United States of America* 46(3): 302–11.
- Collins, Agnieszka, Anthony Warrington, Kenneth A. Taylor, and Tatyana Svitkina. 2011a. 'Structural Organization of the Actin Cytoskeleton at Sites of Clathrin-Mediated Endocytosis'. *Current biology: CB* 21(14): 1167–75.
- Collins, A et al. 2011b. 'Structural Organization of the Actin Cytoskeleton at Sites of Clathrin-Mediated Endocytosis'. *Current biology: CB* 21(14): 1167–75.
- Condeelis, John, and Jeffrey E. Segall. 2003. 'Intravital Imaging of Cell Movement in Tumours'. *Nature Reviews. Cancer* 3(12): 921–30.
- Conner, Sean D., and Sandra L. Schmid. 2003. 'Regulated Portals of Entry into the Cell'. *Nature* 422(6927): 37–44.
- Craig, A. S., M. J. Birtles, J. F. Conway, and D. A. Parry. 1989. 'An Estimate of the Mean Length of Collagen Fibrils in Rat Tail-Tendon as a Function of Age'. *Connective Tissue Research* 19(1): 51–62.
- Cramer, L. P., M. Siebert, and T. J. Mitchison. 1997. 'Identification of Novel Graded Polarity Actin Filament Bundles in Locomoting Heart Fibroblasts: Implications for the Generation of Motile Force'. *The Journal of Cell Biology* 136(6): 1287–1305.
- Creely, J. J. et al. 1990. 'Effects of Epidermal Growth Factor on Collagen Synthesis by an Epithelioid Cell Line Derived from Normal Rat Kidney.' *The American Journal of Pathology* 136(6): 1247–57.
- Cremona, O. et al. 1999. 'Essential Role of Phosphoinositide Metabolism in Synaptic Vesicle Recycling'. *Cell* 99(2): 179–88.
- Crowther, R. A., and B. M. Pearse. 1981. 'Assembly and Packing of Clathrin into Coats.' *The Journal of Cell Biology* 91(3): 790–97.
- Cukierman, E., R. Pankov, D. R. Stevens, and K. M. Yamada. 2001. 'Taking Cell-Matrix Adhesions to the Third Dimension'. *Science (New York, N.Y.)* 294(5547): 1708–12.
- Cybulsky, A. V., A. J. McTavish, and M. D. Cyr. 1994. 'Extracellular Matrix Modulates Epidermal Growth Factor Receptor Activation in Rat Glomerular Epithelial Cells'. *The Journal of Clinical Investigation* 94(1): 68–78.
- Damke, Hanna et al. 2001. 'Dynamin GTPase Domain Mutants Block Endocytic Vesicle Formation at Morphologically Distinct Stages'. *Molecular Biology of the Cell* 12(9): 2578–89.

- Dannhauser, Philip N., and Ernst J. Ungewickell. 2012. 'Reconstitution of Clathrin-Coated Bud and Vesicle Formation with Minimal Components'. *Nature Cell Biology* 14(6): 634–39.
- De Deyne, P. G. et al. 1998. 'The Vitronectin Receptor Associates with Clathrin-Coated Membrane Domains via the Cytoplasmic Domain of Its Beta5 Subunit'. *Journal of Cell Science* 111 (Pt 18): 2729–40.
- De Pascalis, Chiara, and Sandrine Etienne-Manneville. 2017. 'Single and Collective Cell Migration: The Mechanics of Adhesions'. *Molecular Biology of the Cell* 28(14): 1833–46.
- Di Guglielmo, G. M. et al. 1994. 'Compartmentalization of SHC, GRB2 and MSOS, and Hyperphosphorylation of Raf-1 by EGF but Not Insulin in Liver Parenchyma'. *The EMBO journal* 13(18): 4269–77.
- Discher, Dennis E., Paul Janmey, and Yu-Li Wang. 2005. 'Tissue Cells Feel and Respond to the Stiffness of Their Substrate'. *Science (New York, N.Y.)* 310(5751): 1139–43.
- Doyle, Andrew D. et al. 2012. 'Micro-Environmental Control of Cell Migration--Myosin IIA Is Required for Efficient Migration in Fibrillar Environments through Control of Cell Adhesion Dynamics'. *Journal of Cell Science* 125(Pt 9): 2244–56.
- Doyle, A et al. 2015. 'Local 3D Matrix Microenvironment Regulates Cell Migration through Spatiotemporal Dynamics of Contractility-Dependent Adhesions'. *Nature Communications* 6. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4643399/> (October 13, 2019).
- Duchek, P. et al. 2001. 'Guidance of Cell Migration by the Drosophila PDGF/VEGF Receptor'. *Cell* 107(1): 17–26.
- Duchek, P., and P. Rørth. 2001. 'Guidance of Cell Migration by EGF Receptor Signaling during Drosophila Oogenesis'. *Science (New York, N.Y.)* 291(5501): 131–33.
- Eaton, Suzanne, and Fernando Martin-Belmonte. 2014. 'Cargo Sorting in the Endocytic Pathway: A Key Regulator of Cell Polarity and Tissue Dynamics'. *Cold Spring Harbor Perspectives in Biology* 6(10). <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4176004/> (October 14, 2019).
- Eberwein, Philipp et al. 2015. 'Modulation of Focal Adhesion Constituents and Their Down-Stream Events by EGF: On the Cross-Talk of Integrins and Growth Factor Receptors'. *Biochimica Et Biophysica Acta* 1853(10 Pt A): 2183–98.
- Ehrlich, Marcelo et al. 2004. 'Endocytosis by Random Initiation and Stabilization of Clathrin-Coated Pits'. *Cell* 118(5): 591–605.
- Eichel, K., D. Jullié, and M. von Zastrow. 2016. 'β-Arrestin Drives MAP Kinase Signalling from Clathrin-Coated Structures after GPCR Dissociation'. *Nature Cell Biology* 18(3): 303–10.
- Elkhatib, Nadia et al. 2017. 'Tubular Clathrin/AP-2 Lattices Pinch Collagen Fibers to Support 3D Cell Migration'. *Science (New York, N.Y.)* 356(6343).
- Endres, Nicholas F. et al. 2013. 'Conformational Coupling across the Plasma Membrane in Activation of the EGF Receptor'. *Cell* 152(3): 543–56.
- Engler, Adam J., Shamik Sen, H. Lee Sweeney, and Dennis E. Discher. 2006. 'Matrix Elasticity Directs Stem Cell Lineage Specification'. *Cell* 126(4): 677–89.

- Espinoza-Sanchez, Sofia et al. 2018. 'Conformational Changes in Arp2/3 Complex Induced by ATP, WASp-VCA, and Actin Filaments'. *Proceedings of the National Academy of Sciences* 115(37): E8642–51.
- Etienne-Manneville, Sandrine. 2013. 'Microtubules in Cell Migration'. *Annual Review of Cell and Developmental Biology* 29: 471–99.
- Ezratty, Ellen J., Claire Bertaux, Eugene E. Marcantonio, and Gregg G. Gundersen. 2009. 'Clathrin Mediates Integrin Endocytosis for Focal Adhesion Disassembly in Migrating Cells'. *The Journal of Cell Biology* 187(5): 733–47.
- Ezratty, Ellen J., Michael A. Partridge, and Gregg G. Gundersen. 2005. 'Microtubule-Induced Focal Adhesion Disassembly Is Mediated by Dynamin and Focal Adhesion Kinase'. *Nature Cell Biology* 7(6): 581–90.
- Faelber, Katja et al. 2013. 'Oligomerization of Dynamin Superfamily Proteins in Health and Disease'. *Progress in Molecular Biology and Translational Science* 117: 411–43.
- Farndale, R. W., J. J. Sixma, M. J. Barnes, and P. G. de Groot. 2004. 'The Role of Collagen in Thrombosis and Hemostasis'. *Journal of thrombosis and haemostasis: JTH* 2(4): 561–73.
- Ferguson, Shawn M., and Pietro De Camilli. 2012. 'Dynamin, a Membrane-Remodelling GTPase'. *Nature Reviews. Molecular Cell Biology* 13(2): 75–88.
- Fisher, Stephanie A. et al. 2018. 'Photo-Immobilized EGF Chemical Gradients Differentially Impact Breast Cancer Cell Invasion and Drug Response in Defined 3D Hydrogels'. *Biomaterials* 178: 751–66.
- Fotin, Alexander et al. 2004. 'Molecular Model for a Complete Clathrin Lattice from Electron Cryomicroscopy'. *Nature* 432(7017): 573–79.
- Fotin, A et al. 2006. 'Structure Determination of Clathrin Coats to Subnanometer Resolution by Single Particle Cryo-Electron Microscopy'. *Journal of Structural Biology* 156(3): 453–60.
- Fraley, Stephanie I. et al. 2010. 'A Distinctive Role for Focal Adhesion Proteins in Three-Dimensional Cell Motility'. *Nature Cell Biology* 12(6): 598–604.
- Franco, Santos J. et al. 2004. 'Calpain-Mediated Proteolysis of Talin Regulates Adhesion Dynamics'. *Nature Cell Biology* 6(10): 977–83.
- Frantz, Christian et al. 2007. 'Positive Feedback between Cdc42 Activity and H⁺ Efflux by the Na-H Exchanger NHE1 for Polarity of Migrating Cells'. *The Journal of Cell Biology* 179(3): 403–10.
- Frantz, Christian, Kathleen M. Stewart, and Valerie M. Weaver. 2010. 'The Extracellular Matrix at a Glance'. *Journal of Cell Science* 123(Pt 24): 4195–4200.
- Freed, Daniel M. et al. 2017. 'EGFR Ligands Differentially Stabilize Receptor Dimers to Specify Signaling Kinetics'. *Cell* 171(3): 683–695.e18.
- Friedl, Peter, and Katarina Wolf. 2003. 'Tumour-Cell Invasion and Migration: Diversity and Escape Mechanisms'. *Nature Reviews. Cancer* 3(5): 362–74.
- Fujimoto, L. Miya, Robyn Roth, John E. Heuser, and Sandra L. Schmid. 2000. 'Actin Assembly Plays a Variable, but Not Obligatory Role in Receptor-Mediated Endocytosis'. *Traffic* 1(2): 161–71.

- Funamoto, Satoru et al. 2002. 'Spatial and Temporal Regulation of 3-Phosphoinositides by PI 3-Kinase and PTEN Mediates Chemotaxis'. *Cell* 109(5): 611–23.
- Gadella, T. W., and T. M. Jovin. 1995. 'Oligomerization of Epidermal Growth Factor Receptors on A431 Cells Studied by Time-Resolved Fluorescence Imaging Microscopy. A Stereochemical Model for Tyrosine Kinase Receptor Activation'. *The Journal of Cell Biology* 129(6): 1543–58.
- Galbraith, Catherine G., Kenneth M. Yamada, and James A. Galbraith. 2007. 'Polymerizing Actin Fibers Position Integrins Primed to Probe for Adhesion Sites'. *Science (New York, N.Y.)* 315(5814): 992–95.
- Garay, Camilo et al. 2015. 'Epidermal Growth Factor-Stimulated Akt Phosphorylation Requires Clathrin or ErbB2 but Not Receptor Endocytosis'. *Molecular Biology of the Cell* 26(19): 3504–19.
- Garner, Omai B. et al. 2011. 'Stage-Dependent Regulation of Mammary Ductal Branching by Heparan Sulfate and HGF-CMet Signaling'. *Developmental Biology* 355(2): 394–403.
- Garrett, Thomas P. J. et al. 2002. 'Crystal Structure of a Truncated Epidermal Growth Factor Receptor Extracellular Domain Bound to Transforming Growth Factor Alpha'. *Cell* 110(6): 763–73.
- Gerhardt, Holger et al. 2003. 'VEGF Guides Angiogenic Sprouting Utilizing Endothelial Tip Cell Filopodia'. *The Journal of Cell Biology* 161(6): 1163–77.
- Giannone, Grégory, and Michael P. Sheetz. 2006. 'Substrate Rigidity and Force Define Form through Tyrosine Phosphatase and Kinase Pathways'. *Trends in Cell Biology* 16(4): 213–23.
- Goel, Hira Lal et al. 2004. 'Selective Modulation of Type 1 Insulin-like Growth Factor Receptor Signaling and Functions by B1 Integrins'. *The Journal of Cell Biology* 166(3): 407–18.
- Goh, Lai Kuan et al. 2010. 'Multiple Mechanisms Collectively Regulate Clathrin-Mediated Endocytosis of the Epidermal Growth Factor Receptor'. *The Journal of Cell Biology* 189(5): 871–83.
- Goh, Lai Kuan, and Alexander Sorkin. 2013. 'Endocytosis of Receptor Tyrosine Kinases'. *Cold Spring Harbor Perspectives in Biology* 5(5): a017459.
- Goley, Erin D., and Matthew D. Welch. 2006. 'The ARP2/3 Complex: An Actin Nucleator Comes of Age'. *Nature Reviews Molecular Cell Biology* 7(10): 713–26.
- Goode, Bruce L., and Michael J. Eck. 2007. 'Mechanism and Function of Formins in the Control of Actin Assembly'. *Annual Review of Biochemistry* 76: 593–627.
- Göstring, Lovisa. 2011. 'Cellular Studies of HER-Family Specific Affibody Molecules'. <http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-156730> (October 17, 2019).
- Grände, Mats et al. 2002. 'Transforming Growth Factor-Beta and Epidermal Growth Factor Synergistically Stimulate Epithelial to Mesenchymal Transition (EMT) through a MEK-Dependent Mechanism in Primary Cultured Pig Thyrocytes'. *Journal of Cell Science* 115(Pt 22): 4227–36.
- Green, H., and B. Goldberg. 1964. 'COLLAGEN AND CELL PROTEIN SYNTHESIS BY AN ESTABLISHED MAMMALIAN FIBROBLAST LINE'. *Nature* 204: 347–49.

- Greenblatt, Melvin, and Philippe Shuvi. 1968. 'Tumor Angiogenesis: Transfilter Diffusion Studies in the Hamster by the Transparent Chamber Technique'. *JNCI: Journal of the National Cancer Institute* 41(1): 111–24.
- Grimes, M. L. et al. 1996. 'Endocytosis of Activated TrkA: Evidence That Nerve Growth Factor Induces Formation of Signaling Endosomes'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 16(24): 7950–64.
- Gross, J., and D. Kirk. 1958. 'The Heat Precipitation of Collagen from Neutral Salt Solutions: Some Rate-Regulating Factors'. *The Journal of Biological Chemistry* 233(2): 355–60.
- Gurtner, Geoffrey C., Sabine Werner, Yann Barrandon, and Michael T. Longaker. 2008. 'Wound Repair and Regeneration'. *Nature* 453(7193): 314–21.
- Harding, C., J. Heuser, and P. Stahl. 1983. 'Receptor-Mediated Endocytosis of Transferrin and Recycling of the Transferrin Receptor in Rat Reticulocytes'. *The Journal of Cell Biology* 97(2): 329–39.
- von der Hardt, Sophia et al. 2007. 'The Bmp Gradient of the Zebrafish Gastrula Guides Migrating Lateral Cells by Regulating Cell-Cell Adhesion'. *Current biology: CB* 17(6): 475–87.
- Hatten, Mary E. 2002. 'New Directions in Neuronal Migration'. *Science (New York, N.Y.)* 297(5587): 1660–63.
- Haugh, J. M. et al. 1999. 'Internalized Epidermal Growth Factor Receptors Participate in the Activation of P21(Ras) in Fibroblasts'. *The Journal of Biological Chemistry* 274(48): 34350–60.
- Hawryluk, Matthew J. et al. 2006. 'Epsin 1 Is a Polyubiquitin-Selective Clathrin-Associated Sorting Protein'. *Traffic (Copenhagen, Denmark)* 7(3): 262–81.
- Hayes, Susan, Anil Chawla, and Silvia Corvera. 2002. 'TGF β Receptor Internalization into EEA1-Enriched Early Endosomes'. *The Journal of Cell Biology* 158(7): 1239–49.
- Henne, William Mike et al. 2010. 'FCHo Proteins Are Nucleators of Clathrin-Mediated Endocytosis'. *Science (New York, N.Y.)* 328(5983): 1281–84.
- Henry, Anastasia G. et al. 2012. 'Regulation of Endocytic Clathrin Dynamics by Cargo Ubiquitination'. *Developmental Cell* 23(3): 519–32.
- Hentze, Matthias W., Martina U. Muckenthaler, Bruno Galy, and Clara Camaschella. 2010. 'Two to Tango: Regulation of Mammalian Iron Metabolism'. *Cell* 142(1): 24–38.
- Hernandez, Lorena et al. 2009. 'The EGF/CSF-1 Paracrine Invasion Loop Can Be Triggered by Heregulin Beta1 and CXCL12'. *Cancer Research* 69(7): 3221–27.
- Heukers, Raimond et al. 2013. 'Endocytosis of EGFR Requires Its Kinase Activity and N-Terminal Transmembrane Dimerization Motif'. *Journal of Cell Science* 126(Pt 21): 4900–4912.
- Higashiyama, S., J. A. Abraham, and M. Klagsbrun. 1993. 'Heparin-Binding EGF-like Growth Factor Stimulation of Smooth Muscle Cell Migration: Dependence on Interactions with Cell Surface Heparan Sulfate'. *The Journal of Cell Biology* 122(4): 933–40.
- Higgins, Matthew K., and Harvey T. McMahon. 2002. 'Snap-Shots of Clathrin-Mediated Endocytosis.' *Trends in biochemical sciences* 27(5): 257–63.

- Higgs, Henry N., and Thomas D. Pollard. 2000. 'Activation by Cdc42 and Pip2 of Wiskott-Aldrich Syndrome Protein (Wasp) Stimulates Actin Nucleation by Arp2/3 Complex'. *The Journal of Cell Biology* 150(6): 1311–20.
- Hinshaw, J. E., and S. L. Schmid. 1995. 'Dynamain Self-Assembles into Rings Suggesting a Mechanism for Coated Vesicle Budding'. *Nature* 374(6518): 190–92.
- Holmes, Kenneth C., David Popp, Werner Gebhard, and Wolfgang Kabsch. 1990. 'Atomic Model of the Actin Filament'. *Nature* 347(6288): 44–49.
- Höning, Stefan et al. 2005. 'Phosphatidylinositol-(4,5)-Bisphosphate Regulates Sorting Signal Recognition by the Clathrin-Associated Adaptor Complex AP2'. *Molecular Cell* 18(5): 519–31.
- Hotulainen, Pirta, and Pekka Lappalainen. 2006. 'Stress Fibers Are Generated by Two Distinct Actin Assembly Mechanisms in Motile Cells'. *The Journal of Cell Biology* 173(3): 383–94.
- Hu, Dana D., Carlos F. Barbas, and Jeffrey W. Smith. 1996. 'An Allosteric Ca²⁺ Binding Site on the B3-Integrins That Regulates the Dissociation Rate for RGD Ligands'. *Journal of Biological Chemistry* 271(36): 21745–51.
- Huang, Fangtian et al. 2006. 'Differential Regulation of EGF Receptor Internalization and Degradation by Multiubiquitination within the Kinase Domain'. *Molecular Cell* 21(6): 737–48.
- Huang, Fangtian, Anastasia Khvorova, William Marshall, and Alexander Sorkin. 2004. 'Analysis of Clathrin-Mediated Endocytosis of Epidermal Growth Factor Receptor by RNA Interference'. *The Journal of Biological Chemistry* 279(16): 16657–61.
- Humphries, Ashley C., and Michael Way. 2013. 'The Non-Canonical Roles of Clathrin and Actin in Pathogen Internalization, Egress and Spread'. *Nature Reviews Microbiology* 11(8): 551–60.
- Humphries, Jonathan D. et al. 2007. 'Vinculin Controls Focal Adhesion Formation by Direct Interactions with Talin and Actin'. *The Journal of Cell Biology* 179(5): 1043–57.
- Humphries, Jonathan D., Adam Byron, and Martin J. Humphries. 2006. 'Integrin Ligands at a Glance'. *Journal of Cell Science* 119(Pt 19): 3901–3.
- Humphries, M. J. 2000. 'Integrin Structure'. *Biochemical Society Transactions* 28(4): 311–39.
- Hynes, Richard O., and Alexandra Naba. 2012. 'Overview of the Matrisome--an Inventory of Extracellular Matrix Constituents and Functions'. *Cold Spring Harbor Perspectives in Biology* 4(1): a004903.
- Ide, AG. 1939. 'Vascularization of the Brown Pearce Rabbit Epithelioma Transplant as Seen in the Transparent Ear Chamber'. *AJR Am J Roentgenol* 42: 891–99.
- Iijima, Miho, and Peter Devreotes. 2002. 'Tumor Suppressor PTEN Mediates Sensing of Chemoattractant Gradients'. *Cell* 109(5): 599–610.
- Imai, K et al. 1997. 'Degradation of Decorin by Matrix Metalloproteinases: Identification of the Cleavage Sites, Kinetic Analyses and Transforming Growth Factor-Beta1 Release.' *Biochemical Journal* 322(Pt 3): 809–14.
- Insall, Robert H. 2010. 'Understanding Eukaryotic Chemotaxis: A Pseudopod-Centred View'. *Nature Reviews. Molecular Cell Biology* 11(6): 453–58.

- Ishitsuka, Taichi, Tomoki Ikuta, Hiroyoshi Ariga, and Ken-Ichi Matsumoto. 2009. 'Serum Tenascin-X Strongly Binds to Vascular Endothelial Growth Factor'. *Biological & Pharmaceutical Bulletin* 32(6): 1004–11.
- Ivaska, Johanna et al. 1999. 'Integrin A2 β 1 Mediates Isoform-Specific Activation of P38 and Upregulation of Collagen Gene Transcription by a Mechanism Involving the A2 Cytoplasmic Tail'. *The Journal of Cell Biology* 147(2): 401–16.
- Ivaska, Johanna, and Jyrki Heino. 2011. 'Cooperation between Integrins and Growth Factor Receptors in Signaling and Endocytosis'. *Annual Review of Cell and Developmental Biology* 27: 291–320.
- Jacquemet, Guillaume, Hellyeh Hamidi, and Johanna Ivaska. 2015. 'Filopodia in Cell Adhesion, 3D Migration and Cancer Cell Invasion'. *Current Opinion in Cell Biology* 36: 23–31.
- Jahed, Zeinab, Hengameh Shams, Mehrdad Mehrbod, and Mohammad R. K. Mofrad. 2014. 'Mechanotransduction Pathways Linking the Extracellular Matrix to the Nucleus'. *International Review of Cell and Molecular Biology* 310: 171–220.
- Jansen, Karin A. et al. 2018. 'The Role of Network Architecture in Collagen Mechanics'. *Biophysical Journal* 114(11): 2665–78.
- Järveläinen, Hannu et al. 2009. 'Extracellular Matrix Molecules: Potential Targets in Pharmacotherapy'. *Pharmacological Reviews* 61(2): 198–223.
- Jékely, Gáspár, Hsin-Ho Sung, Carlos M. Luque, and Pernille Rørth. 2005. 'Regulators of Endocytosis Maintain Localized Receptor Tyrosine Kinase Signaling in Guided Migration'. *Developmental Cell* 9(2): 197–207.
- Johannessen, Lene E. et al. 2006. 'Activation of the Epidermal Growth Factor (EGF) Receptor Induces Formation of EGF Receptor- and Grb2-Containing Clathrin-Coated Pits'. *Molecular and Cellular Biology* 26(2): 389–401.
- Jost, M. et al. 1998. 'Phosphatidylinositol-4,5-Bisphosphate Is Required for Endocytic Coated Vesicle Formation'. *Current biology: CB* 8(25): 1399–1402.
- Kajanne, Risto et al. 2007. 'EGF-R Regulates MMP Function in Fibroblasts through MAPK and AP-1 Pathways'. *Journal of Cellular Physiology* 212(2): 489–97.
- Kaksonen, Marko, and Aurélien Roux. 2018. 'Mechanisms of Clathrin-Mediated Endocytosis'. *Nature Reviews. Molecular Cell Biology* 19(5): 313–26.
- Kalluri, Raghu, and Robert A. Weinberg. 2009. 'The Basics of Epithelial-Mesenchymal Transition'. *The Journal of Clinical Investigation* 119(6): 1420–28.
- Kanchanawong, Pakorn et al. 2010. 'Nanoscale Architecture of Integrin-Based Cell Adhesions'. *Nature* 468(7323): 580–84.
- Katoh, Kazuo et al. 2001. 'Rho-Kinase-Mediated Contraction of Isolated Stress Fibers'. *The Journal of Cell Biology* 153(3): 569–84.
- Kaverina, Irina, Olga Krylyshkina, and J. Victor Small. 1999. 'Microtubule Targeting of Substrate Contacts Promotes Their Relaxation and Dissociation'. *The Journal of Cell Biology* 146(5): 1033–44.

- Kazazic, Maja et al. 2009. 'Epsin 1 Is Involved in Recruitment of Ubiquitinated EGF Receptors into Clathrin-Coated Pits'. *Traffic (Copenhagen, Denmark)* 10(2): 235–45.
- Kedrin, Dmitriy et al. 2007. 'Cell Motility and Cytoskeletal Regulation in Invasion and Metastasis'. *Journal of Mammary Gland Biology and Neoplasia* 12(2–3): 143–52.
- Keen, James H., Mark C. Willingham, and Ira H. Pastan. 1979. 'Clathrin-Coated Vesicles: Isolation, Dissociation and Factor-Dependent Reassociation of Clathrin Baskets'. *Cell* 16(2): 303–12.
- Keller, H., and P. Eggli. 1998. 'Protrusive Activity, Cytoplasmic Compartmentalization, and Restriction Rings in Locomoting Blebbing Walker Carcinosarcoma Cells Are Related to Detachment of Cortical Actin from the Plasma Membrane'. *Cell Motility and the Cytoskeleton* 41(2): 181–93.
- Kempiak, Stephan J., Shu-Chin Yip, Jonathan M. Backer, and Jeffrey E. Segall. 2003. 'Local Signaling by the EGF Receptor'. *The Journal of Cell Biology* 162(5): 781–88.
- Kim, Daehwan et al. 2015. 'Epidermal Growth Factor Improves the Migration and Contractility of Aged Fibroblasts Cultured on 3D Collagen Matrices'. *International Journal of Molecular Medicine* 35(4): 1017–25.
- Kim, Sangmin et al. 2009. 'EGF-Induced MMP-9 Expression Is Mediated by the JAK3/ERK Pathway, but Not by the JAK3/STAT-3 Pathway in a SKBR3 Breast Cancer Cell Line'. *Cellular Signalling* 21(6): 892–98.
- Kirchhausen, T., and S. C. Harrison. 1981. 'Protein Organization in Clathrin Trimers'. *Cell* 23(3): 755–61.
- Klapholz, Benjamin et al. 2015. 'Alternative Mechanisms for Talin to Mediate Integrin Function'. *Current biology: CB* 25(7): 847–57.
- Knight, C. G. et al. 1998. 'Identification in Collagen Type I of an Integrin Alpha2 Beta1-Binding Site Containing an Essential GER Sequence'. *The Journal of Biological Chemistry* 273(50): 33287–94.
- Kreuger, Johan, Lidia Perez, Antonio J. Giraldez, and Stephen M. Cohen. 2004. 'Opposing Activities of Dally-like Glypican at High and Low Levels of Wingless Morphogen Activity'. *Developmental Cell* 7(4): 503–12.
- Kubow, Kristopher E., and Alan Rick Horwitz. 2011. 'Reducing Background Fluorescence Reveals Adhesions in 3D Matrices'. *Nature Cell Biology* 13(1): 3–5; author reply 5-7.
- Kuo, Wei-Ting et al. 2015. 'Quantitative Analysis of Ligand-EGFR Interactions: A Platform for Screening Targeting Molecules'. *PLoS ONE* 10(2).
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4344348/> (October 18, 2019).
- Kurosaka, Satoshi, and Anna Kashina. 2008. 'Cell Biology of Embryonic Migration'. *Birth Defects Research. Part C, Embryo Today: Reviews* 84(2): 102–22.
- Laato, M., V. M. Kähäri, J. Niinikoski, and E. Vuorio. 1987. 'Epidermal Growth Factor Increases Collagen Production in Granulation Tissue by Stimulation of Fibroblast Proliferation and Not by Activation of Procollagen Genes'. *The Biochemical Journal* 247(2): 385–88.

- Lai, Frank P.L. et al. 2009. 'Cortactin Promotes Migration and Platelet-Derived Growth Factor-Induced Actin Reorganization by Signaling to Rho-GTPases'. *Molecular Biology of the Cell* 20(14): 3209–23.
- Lamaze, C., and S. L. Schmid. 1995. 'Recruitment of Epidermal Growth Factor Receptors into Coated Pits Requires Their Activated Tyrosine Kinase'. *The Journal of Cell Biology* 129(1): 47–54.
- Lambert, Arthur W., Diwakar R. Pattabiraman, and Robert A. Weinberg. 2017. 'Emerging Biological Principles of Metastasis'. *Cell* 168(4): 670–91.
- Lampe, Marko, Stéphane Vassilopoulos, and Christien Merrifield. 2016. 'Clathrin Coated Pits, Plaques and Adhesion'. *Journal of Structural Biology* 196(1): 48–56.
- Lauffenburger, D. A., and A. F. Horwitz. 1996. 'Cell Migration: A Physically Integrated Molecular Process'. *Cell* 84(3): 359–69.
- Laukaitis, C. M., D. J. Webb, K. Donais, and A. F. Horwitz. 2001. 'Differential Dynamics of Alpha 5 Integrin, Paxillin, and Alpha-Actinin during Formation and Disassembly of Adhesions in Migrating Cells'. *The Journal of Cell Biology* 153(7): 1427–40.
- Lawson, M. A., and F. R. Maxfield. 1995. 'Ca(2+)- and Calcineurin-Dependent Recycling of an Integrin to the Front of Migrating Neutrophils'. *Nature* 377(6544): 75–79.
- Le Gall, Sylvain M., Rodolphe Auger, Catherine Dreux, and Philippe Mauduit. 2003. 'Regulated Cell Surface Pro-EGF Ectodomain Shedding Is a Zinc Metalloprotease-Dependent Process'. *The Journal of Biological Chemistry* 278(46): 45255–68.
- Lee, Ho-Sup et al. 2004. 'Characterization of an Actin-Binding Site within the Talin FERM Domain'. *Journal of Molecular Biology* 343(3): 771–84.
- Lee, Juliet. 2018. 'Insights into Cell Motility Provided by the Iterative Use of Mathematical Modeling and Experimentation'. *AIMS Biophysics* 5(2): 97.
- Legerstee, Karin, Bart Geverts, Johan A. Slotman, and Adriaan B. Houtsmuller. 2019. 'Dynamics and Distribution of Paxillin, Vinculin, Zyxin and VASP Depend on Focal Adhesion Location and Orientation'. *Scientific Reports* 9(1): 1–18.
- Leitinger, Birgit, and Erhard Hohenester. 2007. 'Mammalian Collagen Receptors'. *Matrix Biology: Journal of the International Society for Matrix Biology* 26(3): 146–55.
- Lemmon, Mark A., and Joseph Schlessinger. 2010. 'Cell Signaling by Receptor-Tyrosine Kinases'. *Cell* 141(7): 1117–34.
- Lherbette, Michael et al. 2019. 'The AP2 Adaptor Enhances Clathrin Coat Stiffness'. *The FEBS Journal* 0(0). <https://febs.onlinelibrary.wiley.com/doi/abs/10.1111/febs.14961> (October 14, 2019).
- Li, Dong et al. 2015. 'Extended-Resolution Structured Illumination Imaging of Endocytic and Cytoskeletal Dynamics'. *Science* 349(6251): aab3500.
- Li, Rong, and Gregg G. Gundersen. 2008. 'Beyond Polymer Polarity: How the Cytoskeleton Builds a Polarized Cell'. *Nature Reviews. Molecular Cell Biology* 9(11): 860–73.

- Lichtner, R. B. et al. 2001. 'Signaling-Inactive Epidermal Growth Factor Receptor/Ligand Complexes in Intact Carcinoma Cells by Quinazoline Tyrosine Kinase Inhibitors'. *Cancer Research* 61(15): 5790–95.
- Liu, Allen P., François Aguet, Gaudenz Danuser, and Sandra L. Schmid. 2010. 'Local Clustering of Transferrin Receptors Promotes Clathrin-Coated Pit Initiation'. *The Journal of Cell Biology* 191(7): 1381–93.
- Liu, Jiang et al. 2004. 'Ouabain Induces Endocytosis of Plasmalemmal Na/K-ATPase in LLC-PK1 Cells by a Clathrin-Dependent Mechanism'. *Kidney International* 66(1): 227–41.
- Liu, Li et al. 2007. 'Tetraspanin CD151 Promotes Cell Migration by Regulating Integrin Trafficking'. *The Journal of Biological Chemistry* 282(43): 31631–42.
- Liu, S. H., M. L. Wong, C. S. Craik, and F. M. Brodsky. 1995. 'Regulation of Clathrin Assembly and Trimerization Defined Using Recombinant Triskelion Hubs'. *Cell* 83(2): 257–67.
- Lo, C. M., H. B. Wang, M. Dembo, and Y. L. Wang. 2000. 'Cell Movement Is Guided by the Rigidity of the Substrate'. *Biophysical Journal* 79(1): 144–52.
- Lock, John G. et al. 2019. 'Clathrin-Containing Adhesion Complexes'. *The Journal of Cell Biology* 218(7): 2086–95.
- Loerke, Dinah et al. 2009. 'Cargo and Dynamin Regulate Clathrin-Coated Pit Maturation'. *PLOS Biology* 7(3): e1000057.
- Longva, Karianne E. et al. 2002. 'Ubiquitination and Proteasomal Activity Is Required for Transport of the EGF Receptor to Inner Membranes of Multivesicular Bodies'. *The Journal of Cell Biology* 156(5): 843–54.
- Lu, Chafen et al. 2010. 'Structural Evidence for Loose Linkage between Ligand Binding and Kinase Activation in the Epidermal Growth Factor Receptor'. *Molecular and Cellular Biology* 30(22): 5432–43.
- Lu, Pengfei, Ken Takai, Valerie M. Weaver, and Zena Werb. 2011. 'Extracellular Matrix Degradation and Remodeling in Development and Disease'. *Cold Spring Harbor Perspectives in Biology* 3(12).
- Lu, Zhimin, Guoqiang Jiang, Peter Blume-Jensen, and Tony Hunter. 2001. 'Epidermal Growth Factor-Induced Tumor Cell Invasion and Metastasis Initiated by Dephosphorylation and Downregulation of Focal Adhesion Kinase'. *Molecular and Cellular Biology* 21(12): 4016–31.
- Luster, A. D. 1998. 'Chemokines--Chemotactic Cytokines That Mediate Inflammation'. *The New England Journal of Medicine* 338(7): 436–45.
- Lyons, R. M., L. E. Gentry, A. F. Purchio, and H. L. Moses. 1990. 'Mechanism of Activation of Latent Recombinant Transforming Growth Factor Beta 1 by Plasmin'. *The Journal of Cell Biology* 110(4): 1361–67.
- Macia, Eric et al. 2006. 'Dynasore, a Cell-Permeable Inhibitor of Dynamin'. *Developmental Cell* 10(6): 839–50.

- Madshus, Inger Helene, and Espen Stang. 2009. 'Internalization and Intracellular Sorting of the EGF Receptor: A Model for Understanding the Mechanisms of Receptor Trafficking'. *Journal of Cell Science* 122(Pt 19): 3433–39.
- Mai, Anja et al. 2014. 'Distinct C-Met Activation Mechanisms Induce Cell Rounding or Invasion through Pathways Involving Integrins, RhoA and HIP1'. *Journal of Cell Science* 127(Pt 9): 1938–52.
- Martin, Christine, Stine F. Pedersen, Albrecht Schwab, and Christian Stock. 2011. 'Intracellular PH Gradients in Migrating Cells'. *American Journal of Physiology. Cell Physiology* 300(3): C490-495.
- Maupin, P., and T. D. Pollard. 1983. 'Improved Preservation and Staining of HeLa Cell Actin Filaments, Clathrin-Coated Membranes, and Other Cytoplasmic Structures by Tannic Acid-Glutaraldehyde-Saponin Fixation'. *The Journal of Cell Biology* 96(1): 51–62.
- Maurer, Meghan E., and Jonathan A. Cooper. 2006. 'The Adaptor Protein Dab2 Sorts LDL Receptors into Coated Pits Independently of AP-2 and ARH'. *Journal of Cell Science* 119(Pt 20): 4235–46.
- McCUTCHEON, M. 1946. 'Chemotaxis in Leukocytes'. *Physiological Reviews* 26(3): 319–36.
- McMahon, Harvey T., and Emmanuel Boucrot. 2011. 'Molecular Mechanism and Physiological Functions of Clathrin-Mediated Endocytosis'. *Nature Reviews. Molecular Cell Biology* 12(8): 517–33.
- Merrifield, Christien J., Britta Qualmann, Michael M. Kessels, and Wolfhard Almers. 2004. 'Neural Wiskott Aldrich Syndrome Protein (N-WASP) and the Arp2/3 Complex Are Recruited to Sites of Clathrin-Mediated Endocytosis in Cultured Fibroblasts'. *European Journal of Cell Biology* 83(1): 13–18.
- Mettlen, Marcel et al. 2010. 'Cargo- and Adaptor-Specific Mechanisms Regulate Clathrin-Mediated Endocytosis'. *The Journal of Cell Biology* 188(6): 919–33.
- Mettlen, M et al. 2018. 'Regulation of Clathrin-Mediated Endocytosis'. *Annual Review of Biochemistry* 87: 871–96.
- Mettouchi, A. et al. 2001. 'Integrin-Specific Activation of Rac Controls Progression through the G(1) Phase of the Cell Cycle'. *Molecular Cell* 8(1): 115–27.
- Miller, Sharon E. et al. 2015. 'CALM Regulates Clathrin-Coated Vesicle Size and Maturation by Directly Sensing and Driving Membrane Curvature'. *Developmental Cell* 33(2): 163–75.
- Mimura, Yoshihiro et al. 2004. 'Epidermal Growth Factor Induces Fibronectin Expression in Human Dermal Fibroblasts via Protein Kinase C δ Signaling Pathway'. *Journal of Investigative Dermatology* 122(6): 1390–98.
- Minina, Sofia, Michal Reichman-Fried, and Erez Raz. 2007. 'Control of Receptor Internalization, Signaling Level, and Precise Arrival at the Target in Guided Cell Migration'. *Current biology: CB* 17(13): 1164–72.
- Mitchison, T. J., and L. P. Cramer. 1996. 'Actin-Based Cell Motility and Cell Locomotion'. *Cell* 84(3): 371–79.

- Mitra, Satyajit K., Daniel A. Hanson, and David D. Schlaepfer. 2005. 'Focal Adhesion Kinase: In Command and Control of Cell Motility'. *Nature Reviews. Molecular Cell Biology* 6(1): 56–68.
- Miura, Yoshiki, Tsuyoshi Takahashi, Stephanie M. Jung, and Masaaki Moroi. 2002. 'Analysis of the Interaction of Platelet Collagen Receptor Glycoprotein VI (GPVI) with Collagen. A Dimeric Form of GPVI, but Not the Monomeric Form, Shows Affinity to Fibrous Collagen'. *The Journal of Biological Chemistry* 277(48): 46197–204.
- Mizutani, Kiyohito et al. 2002. 'Essential Role of Neural Wiskott-Aldrich Syndrome Protein in Podosome Formation and Degradation of Extracellular Matrix in Src-Transformed Fibroblasts'. *Cancer Research* 62(3): 669–74.
- Montagnac, Guillaume et al. 2013. 'ATAT1 Catalyses Microtubule Acetylation at Clathrin-Coated Pits'. *Nature* 502(7472): 567–70.
- Morelli, Joseph G et al. 1992. 'Leukotriene C4 and TGF-Alpha Are Stimulators of Human Melanocyte Migration In Vitro'. *Journal of Investigative Dermatology* 98(3): 290–95.
- Mori, Hidetoshi et al. 2002. 'CD44 Directs Membrane-Type 1 Matrix Metalloproteinase to Lamellipodia by Associating with Its Hemopexin-like Domain'. *The EMBO Journal* 21(15): 3949–59.
- Moro, L et al. 1998. 'Integrins Induce Activation of EGF Receptor: Role in MAP Kinase Induction and Adhesion-Dependent Cell Survival.' *The EMBO Journal* 17(22): 6622–32.
- Moro, Laura et al. 2002. 'Integrin-Induced Epidermal Growth Factor (EGF) Receptor Activation Requires c-Src and P130Cas and Leads to Phosphorylation of Specific EGF Receptor Tyrosines'. *The Journal of Biological Chemistry* 277(11): 9405–14.
- Motley, Alison, Nicholas A. Bright, Matthew N. J. Seaman, and Margaret S. Robinson. 2003. 'Clathrin-Mediated Endocytosis in AP-2-Depleted Cells'. *The Journal of Cell Biology* 162(5): 909–18.
- Mouneimne, Ghassan et al. 2004. 'Phospholipase C and Cofilin Are Required for Carcinoma Cell Directionality in Response to EGF Stimulation'. *The Journal of Cell Biology* 166(5): 697–708.
- Mouw, Janna K., Guanqing Ou, and Valerie M. Weaver. 2014. 'Extracellular Matrix Assembly: A Multiscale Deconstruction'. *Nature Reviews. Molecular Cell Biology* 15(12): 771–85.
- Mullins, R. Dyche, John A. Heuser, and Thomas D. Pollard. 1998. 'The Interaction of Arp2/3 Complex with Actin: Nucleation, High Affinity Pointed End Capping, and Formation of Branching Networks of Filaments'. *Proceedings of the National Academy of Sciences* 95(11): 6181–86.
- Munevar, Steven, Yu-li Wang, and Micah Dembo. 2001. 'Distinct Roles of Frontal and Rear Cell-Substrate Adhesions in Fibroblast Migration'. *Molecular Biology of the Cell* 12(12): 3947–54.
- Naba, Alexandra et al. 2012. 'The Matrisome: In Silico Definition and in Vivo Characterization by Proteomics of Normal and Tumor Extracellular Matrices'. *Molecular & cellular proteomics: MCP* 11(4): M111.014647.
- Naldini, L. et al. 1992. 'Extracellular Proteolytic Cleavage by Urokinase Is Required for Activation of Hepatocyte Growth Factor/Scatter Factor'. *The EMBO journal* 11(13): 4825–33.

- Nesterov, A et al. 1999. 'Inhibition of the Receptor-Binding Function of Clathrin Adaptor Protein AP-2 by Dominant-Negative Mutant Mu2 Subunit and Its Effects on Endocytosis.' *The EMBO Journal* 18(9): 2489–99.
- Nieselstein-Post, P., G. Mottino, A. Fogelman, and J. Frank. 1994. 'An Ultrastructural Study of Lipoprotein Accumulation in Cardiac Valves of the Rabbit'. *Arteriosclerosis and Thrombosis: A Journal of Vascular Biology* 14(7): 1151–61.
- Niewiadomska, P., D. Godt, and U. Tepass. 1999. 'DE-Cadherin Is Required for Intercellular Motility during *Drosophila* Oogenesis'. *The Journal of Cell Biology* 144(3): 533–47.
- Niggemann, Bernd et al. 1997. 'Locomotory Phenotypes of Human Tumor Cell Lines and T Lymphocytes in a Three-Dimensional Collagen Lattice'. *Cancer Letters* 118(2): 173–80.
- Nishida, Noritaka et al. 2006. 'Activation of Leukocyte Beta2 Integrins by Conversion from Bent to Extended Conformations'. *Immunity* 25(4): 583–94.
- Nurcombe, V., M. D. Ford, J. A. Wildschut, and P. F. Bartlett. 1993. 'Developmental Regulation of Neural Response to FGF-1 and FGF-2 by Heparan Sulfate Proteoglycan'. *Science (New York, N.Y.)* 260(5104): 103–6.
- Ohta, Yasutaka, John H. Hartwig, and Thomas P. Stossel. 2006. 'FilGAP, a Rho- and ROCK-Regulated GAP for Rac Binds Filamin A to Control Actin Remodelling'. *Nature Cell Biology* 8(8): 803–14.
- Oikawa, Tsukasa et al. 2004. 'PtdIns(3,4,5)P3 Binding Is Necessary for WAVE2-Induced Formation of Lamellipodia'. *Nature Cell Biology* 6(5): 420–26.
- Ongusaha, Pat P. et al. 2003. 'P53 Induction and Activation of DDR1 Kinase Counteract P53-Mediated Apoptosis and Influence P53 Regulation through a Positive Feedback Loop'. *The EMBO journal* 22(6): 1289–1301.
- Orgaz, Jose L. et al. 2014. 'Diverse Matrix Metalloproteinase Functions Regulate Cancer Amoeboid Migration'. *Nature Communications* 5(1): 1–13.
- Oser, Matthew, and John Condeelis. 2009. 'The Cofilin Activity Cycle in Lamellipodia and Invadopodia'. *Journal of Cellular Biochemistry* 108(6): 1252–62.
- Oudin, Madeleine J. et al. 2016. 'Tumor Cell-Driven Extracellular Matrix Remodeling Drives Haptotaxis during Metastatic Progression'. *Cancer Discovery* 6(5): 516–31.
- Palecek, S. P., C. E. Schmidt, D. A. Lauffenburger, and A. F. Horwitz. 1996. 'Integrin Dynamics on the Tail Region of Migrating Fibroblasts'. *Journal of Cell Science* 109 (Pt 5): 941–52.
- Paralkar, Vishwas M., Slobodan Vukicevic, and A. H. Reddi. 1991. 'Transforming Growth Factor β Type 1 Binds to Collagen IV of Basement Membrane Matrix: Implications for Development'. *Developmental Biology* 143(2): 303–8.
- Parent, C. A., and P. N. Devreotes. 1999. 'A Cell's Sense of Direction'. *Science (New York, N.Y.)* 284(5415): 765–70.
- Parkin, J., and B. Cohen. 2001. 'An Overview of the Immune System'. *Lancet (London, England)* 357(9270): 1777–89.

- Pascolutti, Roberta et al. 2019. 'Molecularly Distinct Clathrin-Coated Pits Differentially Impact EGFR Fate and Signaling'. *Cell Reports* 27(10): 3049-3061.e6.
- Pearlstein, Edward. 1976. 'Plasma Membrane Glycoprotein Which Mediates Adhesion of Fibroblasts to Collagen'. *Nature* 262(5568): 497-500.
- Pentikäinen, M. O., K. Oörni, R. Lassila, and P. T. Kovanen. 1997. 'The Proteoglycan Decorin Links Low Density Lipoproteins with Collagen Type I'. *The Journal of Biological Chemistry* 272(12): 7633-38.
- Peskin, C S, G M Odell, and G F Oster. 1993. 'Cellular Motions and Thermal Fluctuations: The Brownian Ratchet'. *Biophysical Journal* 65(1): 316-24.
- Peter, Brian J. et al. 2004. 'BAR Domains as Sensors of Membrane Curvature: The Amphiphysin BAR Structure'. *Science (New York, N.Y.)* 303(5657): 495-99.
- Petitclerc, E. et al. 1999. 'Integrin Alpha(v)Beta3 Promotes M21 Melanoma Growth in Human Skin by Regulating Tumor Cell Survival'. *Cancer Research* 59(11): 2724-30.
- Pietu, G. et al. 1987. 'Binding of Human von Willebrand Factor to Collagen and to Collagen-Stimulated Platelets'. *The Journal of Laboratory and Clinical Medicine* 109(6): 637-46.
- Pilcher, B. K. et al. 1997. 'The Activity of Collagenase-1 Is Required for Keratinocyte Migration on a Type I Collagen Matrix'. *The Journal of Cell Biology* 137(6): 1445-57.
- Pilcher, Brian K. et al. 1999. 'Keratinocyte Collagenase-1 Expression Requires an Epidermal Growth Factor Receptor Autocrine Mechanism'. *Journal of Biological Chemistry* 274(15): 10372-81.
- Platt, Manu O. et al. 2009. 'Sustained Epidermal Growth Factor Receptor Levels and Activation by Tethered Ligand Binding Enhances Osteogenic Differentiation of Multi-Potent Marrow Stromal Cells'. *Journal of Cellular Physiology* 221(2): 306-17.
- Poincloux, Renaud et al. 2011. 'Contractility of the Cell Rear Drives Invasion of Breast Tumor Cells in 3D Matrigel'. *Proceedings of the National Academy of Sciences* 108(5): 1943-48.
- Pollard, Thomas D., and Gary G. Borisy. 2003. 'Cellular Motility Driven by Assembly and Disassembly of Actin Filaments'. *Cell* 112(4): 453-65.
- Pollard, Thomas D., and John A. Cooper. 1984. 'Quantitative Analysis of the Effect of Acanthamoeba Profilin on Actin Filament Nucleation and Elongation'. *Biochemistry* 23(26): 6631-41.
- Prager-Khoutorsky, Masha et al. 2011. 'Fibroblast Polarization Is a Matrix-Rigidity-Dependent Process Controlled by Focal Adhesion Mechanosensing'. *Nature Cell Biology* 13(12): 1457-65.
- Pucadyil, Thomas J., and Sachin S. Holkar. 2016. 'Comparative Analysis of Adaptor-Mediated Clathrin Assembly Reveals General Principles for Adaptor Clustering'. *Molecular Biology of the Cell* 27(20): 3156-63.
- Qualmann, Britta, Dennis Koch, and Michael Manfred Kessels. 2011. 'Let's Go Bananas: Revisiting the Endocytic BAR Code'. *The EMBO Journal* 30(17): 3501-15.
- Quinlan, Margot E., John E. Heuser, Eugen Kerkhoff, and R. Dyche Mullins. 2005. 'Drosophila Spire Is an Actin Nucleation Factor'. *Nature* 433(7024): 382-88.

- Rahman, Salman et al. 2005. 'Novel Hepatocyte Growth Factor (HGF) Binding Domains on Fibronectin and Vitronectin Coordinate a Distinct and Amplified Met-Integrin Induced Signalling Pathway in Endothelial Cells'. *BMC cell biology* 6(1): 8.
- Raja, Waseem Khan et al. 2010. 'A New Chemotaxis Device for Cell Migration Studies'. *Integrative biology : quantitative biosciences from nano to macro* 2(0): 696–706.
- Rappoport, Joshua Z., and Sanford M. Simon. 2009. 'Endocytic Trafficking of Activated EGFR Is AP-2 Dependent and Occurs through Preformed Clathrin Spots'. *Journal of Cell Science* 122(Pt 9): 1301–5.
- Raub, Thomas J., and Sandra L. Kuentzel. 1989. 'Kinetic and Morphological Evidence for Endocytosis of Mammalian Cell Integrin Receptors by Using an Anti-Fibronectin Receptor β Subunit Monoclonal Antibody'. *Experimental Cell Research* 184(2): 407–26.
- Rayment, I. et al. 1993. 'Structure of the Actin-Myosin Complex and Its Implications for Muscle Contraction'. *Science (New York, N.Y.)* 261(5117): 58–65.
- Raynal, Nicolas et al. 2006. 'Use of Synthetic Peptides to Locate Novel Integrin Alpha2beta1-Binding Motifs in Human Collagen III'. *The Journal of Biological Chemistry* 281(7): 3821–31.
- Regen, C. M., and A. F. Horwitz. 1992. 'Dynamics of Beta 1 Integrin-Mediated Adhesive Contacts in Motile Fibroblasts'. *The Journal of Cell Biology* 119(5): 1347–59.
- Reig, Germán, Eduardo Pulgar, and Miguel L. Concha. 2014. 'Cell Migration: From Tissue Culture to Embryos'. *Development (Cambridge, England)* 141(10): 1999–2013.
- Reyhani, Vahid et al. 2014. 'Fibrin Binds to Collagen and Provides a Bridge for AV β 3 Integrin-Dependent Contraction of Collagen Gels'. *The Biochemical Journal* 462(1): 113–23.
- Rickert, Paula et al. 2000. 'Leukocytes Navigate by Compass: Roles of PI3K γ and Its Lipid Products'. *Trends in cell biology* 10(11): 466.
- Rijken, P. J. et al. 1991. 'Epidermal Growth Factor Induces Rapid Reorganization of the Actin Microfilament System in Human A431 Cells'. *Journal of Cell Science* 100 (Pt 3): 491–99.
- del Rio, Armando et al. 2009. 'Stretching Single Talin Rod Molecules Activates Vinculin Binding'. *Science (New York, N.Y.)* 323(5914): 638–41.
- Rohatgi, R. et al. 1999. 'The Interaction between N-WASP and the Arp2/3 Complex Links Cdc42-Dependent Signals to Actin Assembly'. *Cell* 97(2): 221–31.
- Roskoski, Robert. 2014. 'The ErbB/HER Family of Protein-Tyrosine Kinases and Cancer'. *Pharmacological Research* 79: 34–74.
- Rouiller, Isabelle et al. 2008. 'The Structural Basis of Actin Filament Branching by the Arp2/3 Complex'. *The Journal of Cell Biology* 180(5): 887–95.
- Rousseau, S. et al. 2000. 'Vascular Endothelial Growth Factor (VEGF)-Driven Actin-Based Motility Is Mediated by VEGFR2 and Requires Concerted Activation of Stress-Activated Protein Kinase 2 (SAPK2/P38) and Geldanamycin-Sensitive Phosphorylation of Focal Adhesion Kinase'. *The Journal of Biological Chemistry* 275(14): 10661–72.

- Rust, Michael J., Melike Lakadamyali, Feng Zhang, and Xiaowei Zhuang. 2004. 'Assembly of Endocytic Machinery around Individual Influenza Viruses during Viral Entry'. *Nature Structural & Molecular Biology* 11(6): 567–73.
- Sabeh, Farideh et al. 2004. 'Tumor Cell Traffic through the Extracellular Matrix Is Controlled by the Membrane-Anchored Collagenase MT1-MMP'. *The Journal of Cell Biology* 167(4): 769–81.
- Saffarian, Saveez, Emanuele Cocucci, and Tomas Kirchhausen. 2009. 'Distinct Dynamics of Endocytic Clathrin-Coated Pits and Coated Plaques'. *PLOS Biology* 7(9): e1000191.
- Saleem, Mohammed et al. 2015. 'A Balance between Membrane Elasticity and Polymerization Energy Sets the Shape of Spherical Clathrin Coats'. *Nature Communications* 6(1): 1–10.
- Sarrazin, Stephane, William C. Lamanna, and Jeffrey D. Esko. 2011. 'Heparan Sulfate Proteoglycans'. *Cold Spring Harbor Perspectives in Biology* 3(7).
<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3119907/> (October 13, 2019).
- Sasaki, T. et al. 2000. 'Function of PI3Kgamma in Thymocyte Development, T Cell Activation, and Neutrophil Migration'. *Science (New York, N.Y.)* 287(5455): 1040–46.
- Saunders, Ruth M. et al. 2006. 'Role of Vinculin in Regulating Focal Adhesion Turnover'. *European Journal of Cell Biology* 85(6): 487–500.
- Saxena, Mayur et al. 2017. 'EGFR and HER2 Activate Rigidity Sensing Only on Rigid Matrices'. *Nature Materials* 16(7): 775–81.
- Schaller, Michael D. 2010. 'Cellular Functions of FAK Kinases: Insight into Molecular Mechanisms and Novel Functions'. *Journal of Cell Science* 123(Pt 7): 1007–13.
- Schenk, Susann et al. 2003. 'Binding to EGF Receptor of a Laminin-5 EGF-like Fragment Liberated during MMP-Dependent Mammary Gland Involution'. *The Journal of Cell Biology* 161(1): 197–209.
- Schlossman, D. M., S. L. Schmid, W. A. Braell, and J. E. Rothman. 1984. 'An Enzyme That Removes Clathrin Coats: Purification of an Uncoating ATPase'. *The Journal of Cell Biology* 99(2): 723–33.
- Schmid, Eva M., and Harvey T. McMahon. 2007. 'Integrating Molecular and Network Biology to Decode Endocytosis'. *Nature* 448(7156): 883–88.
- Schmidt, C. E., A. F. Horwitz, D. A. Lauffenburger, and M. P. Sheetz. 1993. 'Integrin-Cytoskeletal Interactions in Migrating Fibroblasts Are Dynamic, Asymmetric, and Regulated'. *The Journal of Cell Biology* 123(4): 977–91.
- Schneller, M., K. Vuori, and E. Ruoslahti. 1997. 'Alphavbeta3 Integrin Associates with Activated Insulin and PDGFbeta Receptors and Potentiates the Biological Activity of PDGF'. *The EMBO journal* 16(18): 5600–5607.
- Schuppan, D. et al. 1998. 'Collagens in the Liver Extracellular Matrix Bind Hepatocyte Growth Factor'. *Gastroenterology* 114(1): 139–52.
- Scita, Giorgio, and Pier Paolo Di Fiore. 2010. 'The Endocytic Matrix'. *Nature* 463(7280): 464–73.

- Scott, Brandon L. et al. 2018. 'Membrane Bending Occurs at All Stages of Clathrin-Coat Assembly and Defines Endocytic Dynamics'. *Nature Communications* 9(1): 1–9.
- Scott, Mark G. H., Alexandre Benmerah, Olivier Muntaner, and Stefano Marullo. 2002. 'Recruitment of Activated G Protein-Coupled Receptors to Pre-Existing Clathrin-Coated Pits in Living Cells'. *The Journal of Biological Chemistry* 277(5): 3552–59.
- Segall, Jeffrey E. et al. 1996. 'EGF Stimulates Lamellipod Extension in Metastatic Mammary Adenocarcinoma Cells by an Actin-Dependent Mechanism'. *Clinical & Experimental Metastasis* 14(1): 61–72.
- Sergina, Natalia V., and Mark M. Moasser. 2007. 'The HER Family and Cancer: Emerging Molecular Mechanisms and Therapeutic Targets'. *Trends in Molecular Medicine* 13(12): 527–34.
- Shemesh, Tom et al. 2009. 'Role of Focal Adhesions and Mechanical Stresses in the Formation and Progression of the Lamellum Interface'. *Biophysical Journal* 97(5): 1254–64.
- Sheppard, D. 2000. 'In Vivo Functions of Integrins: Lessons from Null Mutations in Mice'. *Matrix Biology: Journal of the International Society for Matrix Biology* 19(3): 203–9.
- Shih, W., A. Gallusser, and T. Kirchhausen. 1995. 'A Clathrin-Binding Site in the Hinge of the Beta 2 Chain of Mammalian AP-2 Complexes'. *The Journal of Biological Chemistry* 270(52): 31083–90.
- Short, Sarah M., Gregory A. Talbott, and Rudolph L. Juliano. 1998. 'Integrin-Mediated Signaling Events in Human Endothelial Cells'. *Molecular Biology of the Cell* 9(8): 1969–80.
- Shrivastava, A. et al. 1997. 'An Orphan Receptor Tyrosine Kinase Family Whose Members Serve as Nonintegrin Collagen Receptors'. *Molecular Cell* 1(1): 25–34.
- Sigismund, Sara et al. 2005. 'Clathrin-Independent Endocytosis of Ubiquitinated Cargos'. *Proceedings of the National Academy of Sciences of the United States of America* 102(8): 2760–65.
- Sigismund, S et al. 2008. 'Clathrin-Mediated Internalization Is Essential for Sustained EGFR Signaling but Dispensable for Degradation'. *Developmental Cell* 15(2): 209–19.
- Smith, Michael L. et al. 2007. 'Force-Induced Unfolding of Fibronectin in the Extracellular Matrix of Living Cells'. *PLoS biology* 5(10): e268.
- Somasundaram, R., and D. Schuppan. 1996. 'Type I, II, III, IV, V, and VI Collagens Serve as Extracellular Ligands for the Isoforms of Platelet-Derived Growth Factor (AA, BB, and AB)'. *The Journal of Biological Chemistry* 271(43): 26884–91.
- Song, Byeong Doo, Marilyn Leonard, and Sandra L. Schmid. 2004. 'Dynamin GTPase Domain Mutants That Differentially Affect GTP Binding, GTP Hydrolysis, and Clathrin-Mediated Endocytosis'. *The Journal of Biological Chemistry* 279(39): 40431–36.
- Sorkin, A. et al. 1991. 'Recycling of Epidermal Growth Factor-Receptor Complexes in A431 Cells: Identification of Dual Pathways'. *The Journal of Cell Biology* 112(1): 55–63.
- Sorkin, A et al. 1996. 'Epidermal Growth Factor Receptor Interaction with Clathrin Adaptors Is Mediated by the Tyr974-Containing Internalization Motif'. *The Journal of Biological Chemistry* 271(23): 13377–84.

- Sorkina, Tatiana, Fangtian Huang, Laura Beguinot, and Alexander Sorkin. 2002. 'Effect of Tyrosine Kinase Inhibitors on Clathrin-Coated Pit Recruitment and Internalization of Epidermal Growth Factor Receptor'. *The Journal of Biological Chemistry* 277(30): 27433–41.
- Sridhar, S. C., and C. K. Miranti. 2006. 'Tetraspanin KAI1/CD82 Suppresses Invasion by Inhibiting Integrin-Dependent Crosstalk with c-Met Receptor and Src Kinases'. *Oncogene* 25(16): 2367–78.
- Steketee, Michael B., and Kathryn W. Tosney. 2002. 'Three Functionally Distinct Adhesions in Filopodia: Shaft Adhesions Control Lamellar Extension'. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience* 22(18): 8071–83.
- Suzuki, M. et al. 1997. 'Matrix Metalloproteinase-3 Releases Active Heparin-Binding EGF-like Growth Factor by Cleavage at a Specific Juxtamembrane Site'. *The Journal of Biological Chemistry* 272(50): 31730–37.
- Svitkina, T. M., and G. G. Borisy. 1999. 'Arp2/3 Complex and Actin Depolymerizing Factor/Cofilin in Dendritic Organization and Treadmilling of Actin Filament Array in Lamellipodia'. *The Journal of Cell Biology* 145(5): 1009–26.
- Svitkina, T. M., A. B. Verkhovsky, K. M. McQuade, and G. G. Borisy. 1997. 'Analysis of the Actin-Myosin II System in Fish Epidermal Keratocytes: Mechanism of Cell Body Translocation'. *The Journal of Cell Biology* 139(2): 397–415.
- Swaney, Kristen F., Chuan-Hsiang Huang, and Peter N. Devreotes. 2010. 'Eukaryotic Chemotaxis: A Network of Signaling Pathways Controls Motility, Directional Sensing, and Polarity'. *Annual Review of Biophysics* 39: 265–89.
- Sweeney, Shawn M. et al. 2008. 'Candidate Cell and Matrix Interaction Domains on the Collagen Fibril, the Predominant Protein of Vertebrates'. *The Journal of Biological Chemistry* 283(30): 21187–97.
- Takei, K., V. I. Slepnev, V. Haucke, and P. De Camilli. 1999. 'Functional Partnership between Amphiphysin and Dynamin in Clathrin-Mediated Endocytosis'. *Nature Cell Biology* 1(1): 33–39.
- Tarbashevich, Katsiaryna, Michal Reichman-Fried, Cecilia Grimaldi, and Erez Raz. 2015. 'Chemokine-Dependent PH Elevation at the Cell Front Sustains Polarity in Directionally Migrating Zebrafish Germ Cells'. *Current biology: CB* 25(8): 1096–1103.
- Tawil, N., P. Wilson, and S. Carbonetto. 1993. 'Integrins in Point Contacts Mediate Cell Spreading: Factors That Regulate Integrin Accumulation in Point Contacts vs. Focal Contacts'. *The Journal of Cell Biology* 120(1): 261–71.
- Taylor, Marcus J., David Perrais, and Christien J. Merrifield. 2011. 'A High Precision Survey of the Molecular Dynamics of Mammalian Clathrin-Mediated Endocytosis'. *PLOS Biology* 9(3): e1000604.
- Tojkander, Sari et al. 2011. 'A Molecular Pathway for Myosin II Recruitment to Stress Fibers'. *Current biology: CB* 21(7): 539–50.
- Tojkander, Sari, Gergana Gateva, and Pekka Lappalainen. 2012. 'Actin Stress Fibers--Assembly, Dynamics and Biological Roles'. *Journal of Cell Science* 125(Pt 8): 1855–64.

- Tran, Andy Dong-Anh et al. 2007. 'HDAC6 Deacetylation of Tubulin Modulates Dynamics of Cellular Adhesions'. *Journal of Cell Science* 120(Pt 8): 1469–79.
- Ullrich, A., and J. Schlessinger. 1990. 'Signal Transduction by Receptors with Tyrosine Kinase Activity'. *Cell* 61(2): 203–12.
- Ulrich, Florian, and Carl-Philipp Heisenberg. 2009. 'Trafficking and Cell Migration'. *Traffic (Copenhagen, Denmark)* 10(7): 811–18.
- Ungewickell, E., and H. Ungewickell. 1991. 'Bovine Brain Clathrin Light Chains Impede Heavy Chain Assembly in Vitro'. *The Journal of Biological Chemistry* 266(19): 12710–14.
- Ungewickell, Ernst, and Daniel Branton. 1981. 'Assembly Units of Clathrin Coats'. *Nature* 289(5796): 420–22.
- Velling, Teet, Anne Stefansson, and Staffan Johansson. 2008. 'EGFR and B1 Integrins Utilize Different Signaling Pathways to Activate Akt'. *Experimental Cell Research* 314(2): 309–16.
- Vicente-Manzanares, Miguel et al. 2007. 'Regulation of Protrusion, Adhesion Dynamics, and Polarity by Myosins IIA and IIB in Migrating Cells'. *The Journal of Cell Biology* 176(5): 573–80.
- Vicente-Manzanares, Miguel, and Alan Rick Horwitz. 2011. 'Cell Migration: An Overview'. *Methods in Molecular Biology (Clifton, N.J.)* 769: 1–24.
- Vicente-Manzanares, Miguel, Xuefei Ma, Robert S. Adelstein, and Alan Rick Horwitz. 2009. 'Non-Muscle Myosin II Takes Centre Stage in Cell Adhesion and Migration'. *Nature Reviews. Molecular Cell Biology* 10(11): 778–90.
- Vieira, Amandio V., Christophe Lamaze, and Sandra L. Schmid. 1996. 'Control of EGF Receptor Signaling by Clathrin-Mediated Endocytosis'. *Science* 274(5295): 2086–89.
- Villaseñor, Roberto et al. 2015. 'Regulation of EGFR Signal Transduction by Analogue-to-Digital Conversion in Endosomes' ed. Suzanne R Pfeffer. *eLife* 4: e06156.
- Vogel, Viola. 2006. 'MECHANOTRANSDUCTION INVOLVING MULTIMODULAR PROTEINS: Converting Force into Biochemical Signals'. *Annual Review of Biophysics and Biomolecular Structure* 35(1): 459–88.
- Vogel, W., G. D. Gish, F. Alves, and T. Pawson. 1997. 'The Discoidin Domain Receptor Tyrosine Kinases Are Activated by Collagen'. *Molecular Cell* 1(1): 13–23.
- Vorotnikov, A. V. 2011. 'Chemotaxis: Movement, Direction, Control'. *Biochemistry. Biokhimiia* 76(13): 1528–55.
- Wang, Chau-Zen et al. 2006. 'A Discoidin Domain Receptor 1/SHP-2 Signaling Complex Inhibits A2 β 1-Integrin-Mediated Signal Transducers and Activators of Transcription 1/3 Activation and Cell Migration'. *Molecular Biology of the Cell* 17(6): 2839–52.
- Wang, Qian, Xinmei Chen, and Zhixiang Wang. 2015. 'Dimerization Drives EGFR Endocytosis through Two Sets of Compatible Endocytic Codes'. *Journal of Cell Science* 128(5): 935–50.
- Wang, Qian, Greg Villeneuve, and Zhixiang Wang. 2005. 'Control of Epidermal Growth Factor Receptor Endocytosis by Receptor Dimerization, Rather than Receptor Kinase Activation'. *EMBO reports* 6(10): 942–48.

- Wang, Shur-Jen et al. 2004. 'Differential Effects of EGF Gradient Profiles on MDA-MB-231 Breast Cancer Cell Chemotaxis'. *Experimental Cell Research* 300(1): 180–89.
- Wang, Weigang, Robert Eddy, and John Condeelis. 2007. 'The Cofilin Pathway in Breast Cancer Invasion and Metastasis'. *Nature reviews. Cancer* 7(6): 429–40.
- Warren, G, J Davoust, and A Cockcroft. 1984. 'Recycling of Transferrin Receptors in A431 Cells Is Inhibited during Mitosis.' *The EMBO Journal* 3(10): 2217–25.
- Watanabe, N. et al. 1997. 'P140mDia, a Mammalian Homolog of Drosophila Diaphanous, Is a Target Protein for Rho Small GTPase and Is a Ligand for Profilin'. *The EMBO journal* 16(11): 3044–56.
- Weber, Michele et al. 2013. 'Interstitial Dendritic Cell Guidance by Haptotactic Chemokine Gradients'. *Science* 339(6117): 328–32.
- Wegener, Kate L. et al. 2007. 'Structural Basis of Integrin Activation by Talin'. *Cell* 128(1): 171–82.
- Wells, Rebecca G. 2008. 'The Role of Matrix Stiffness in Regulating Cell Behavior'. *Hepatology* 47(4): 1394–1400.
- Westermarck, B., A. Magnusson, and C. H. Heldin. 1982. 'Effect of Epidermal Growth Factor on Membrane Motility and Cell Locomotion in Cultures of Human Clonal Glioma Cells'. *Journal of Neuroscience Research* 8(2–3): 491–507.
- von Wichert, Götz, Beatrice Haimovich, Gen-Sheng Feng, and Michael P. Sheetz. 2003. 'Force-Dependent Integrin–Cytoskeleton Linkage Formation Requires Downregulation of Focal Complex Dynamics by Shp2'. *The EMBO Journal* 22(19): 5023–35.
- Wijelath, Errol S. et al. 2006. 'Heparin-II Domain of Fibronectin Is a Vascular Endothelial Growth Factor-Binding Domain: Enhancement of VEGF Biological Activity by a Singular Growth Factor/Matrix Protein Synergism'. *Circulation Research* 99(8): 853–60.
- Wilde, A. et al. 1999. 'EGF Receptor Signaling Stimulates SRC Kinase Phosphorylation of Clathrin, Influencing Clathrin Redistribution and EGF Uptake'. *Cell* 96(5): 677–87.
- Wilkins-Port, Cynthia E., and Paul J. Higgins. 2007. 'Regulation of Extracellular Matrix Remodeling Following Transforming Growth Factor-Beta1/Epidermal Growth Factor-Stimulated Epithelial-Mesenchymal Transition in Human Premalignant Keratinocytes'. *Cells, Tissues, Organs* 185(1–3): 116–22.
- Winograd-Katz, Sabina E., Reinhard Fässler, Benjamin Geiger, and Kyle R. Legate. 2014. 'The Integrin Adhesome: From Genes and Proteins to Human Disease'. *Nature Reviews. Molecular Cell Biology* 15(4): 273–88.
- Wise, Steven G., and Anthony S. Weiss. 2009. 'Tropoelastin'. *The International Journal of Biochemistry & Cell Biology* 41(3): 494–97.
- Wolf, Katarina et al. 2003. 'Compensation Mechanism in Tumor Cell Migration: Mesenchymal-Amoeboid Transition after Blocking of Pericellular Proteolysis'. *The Journal of Cell Biology* 160(2): 267–77.
- Wood, G. C., and M. K. Keech. 1960. 'The Formation of Fibrils from Collagen Solutions. 1. The Effect of Experimental Conditions: Kinetic and Electron-Microscope Studies'. *The Biochemical Journal* 75: 588–98.

- Wu, Congying et al. 2012. 'Arp2/3 Is Critical for Lamellipodia and Response to Extracellular Matrix Cues but Is Dispensable for Chemotaxis'. *Cell* 148(5): 973–87.
- Wu, Ning et al. 2013. 'AMPK-Dependent Degradation of TXNIP upon Energy Stress Leads to Enhanced Glucose Uptake via GLUT1'. *Molecular Cell* 49(6): 1167–75.
- Wyckoff, Jeffrey et al. 2004. 'A Paracrine Loop between Tumor Cells and Macrophages Is Required for Tumor Cell Migration in Mammary Tumors'. *Cancer Research* 64(19): 7022–29.
- Xie, H. et al. 1998. 'EGF Receptor Regulation of Cell Motility: EGF Induces Disassembly of Focal Adhesions Independently of the Motility-Associated PLCgamma Signaling Pathway'. *Journal of Cell Science* 111 (Pt 5): 615–24.
- Xiong, Yuan, Chuan-Hsiang Huang, Pablo A. Iglesias, and Peter N. Devreotes. 2010. 'Cells Navigate with a Local-Excitation, Global-Inhibition-Biased Excitable Network'. *Proceedings of the National Academy of Sciences* 107(40): 17079–86.
- Xu, Y. et al. 2000. 'Multiple Binding Sites in Collagen Type I for the Integrins Alpha1beta1 and Alpha2beta1'. *The Journal of Biological Chemistry* 275(50): 38981–89.
- Xue, Chengsen et al. 2006. 'Epidermal Growth Factor Receptor Overexpression Results in Increased Tumor Cell Motility in Vivo Coordinately with Enhanced Intravasation and Metastasis'. *Cancer Research* 66(1): 192–97.
- Yamada, Kenneth M., and Michael Sixt. 2019. 'Mechanisms of 3D Cell Migration'. *Nature Reviews. Molecular Cell Biology*.
- Yamaguchi, Hideki, and John Condeelis. 2007. 'Regulation of the Actin Cytoskeleton in Cancer Cell Migration and Invasion'. *Biochimica Et Biophysica Acta* 1773(5): 642–52.
- Yang, Changsong, and Tatyana Svitkina. 2011. 'Filopodia Initiation: Focus on the Arp2/3 Complex and Formins'. *Cell Adhesion & Migration* 5(5): 402–8.
- Yang, Chin-Chiang, Sin-Daw Lin, and Hsin-Su Yu. 1997. 'Effect of Growth Factors on Dermal Fibroblast Contraction in Normal Skin and Hypertrophic Scar'. *Journal of Dermatological Science* 14(2): 162–69.
- Yang, Yifan et al. 2009. 'Collagen-Binding Human Epidermal Growth Factor Promotes Cellularization of Collagen Scaffolds'. *Tissue Engineering. Part A* 15(11): 3589–96.
- Yarwood, S. J., and J. R. Woodgett. 2001. 'Extracellular Matrix Composition Determines the Transcriptional Response to Epidermal Growth Factor Receptor Activation'. *Proceedings of the National Academy of Sciences of the United States of America* 98(8): 4472–77.
- Yin, Helen L., and Paul A. Janmey. 2003. 'Phosphoinositide Regulation of the Actin Cytoskeleton'. *Annual Review of Physiology* 65: 761–89.
- Yumura, S., H. Mori, and Y. Fukui. 1984. 'Localization of Actin and Myosin for the Study of Ameboid Movement in Dictyostelium Using Improved Immunofluorescence.' *The Journal of Cell Biology* 99(3): 894–99.
- Zaman, Muhammad H. et al. 2006. 'Migration of Tumor Cells in 3D Matrices Is Governed by Matrix Stiffness along with Cell-Matrix Adhesion and Proteolysis'. *Proceedings of the National Academy of Sciences of the United States of America* 103(29): 10889–94.

- Zhang, J. et al. 1999. 'Cellular Trafficking of G Protein-Coupled Receptor/Beta-Arrestin Endocytic Complexes'. *The Journal of Biological Chemistry* 274(16): 10999–6.
- Zhang, Xiao-Feng et al. 2003. 'Rho-Dependent Contractile Responses in the Neuronal Growth Cone Are Independent of Classical Peripheral Retrograde Actin Flow'. *Neuron* 40(5): 931–44.
- Zhang, Xuewu et al. 2006. 'An Allosteric Mechanism for Activation of the Kinase Domain of Epidermal Growth Factor Receptor'. *Cell* 125(6): 1137–49.
- Zhao, M., A. Agius-Fernandez, J. V. Forrester, and C. D. McCaig. 1996. 'Orientation and Directed Migration of Cultured Corneal Epithelial Cells in Small Electric Fields Are Serum Dependent'. *Journal of Cell Science* 109 (Pt 6): 1405–14.
- Zheng, J. et al. 1996. 'Identification of the Binding Site for Acidic Phospholipids on the PH Domain of Dynamin: Implications for Stimulation of GTPase Activity'. *Journal of Molecular Biology* 255(1): 14–21.
- Zuidema, Alba et al. 2018. 'Mechanisms of Integrin AV β 5 Clustering in Flat Clathrin Lattices'. *Journal of Cell Science* 131(21): jcs221317.

RESUME LONG EN FRANÇAIS

La migration cellulaire est un mécanisme fondamental qui se produit pendant toute la vie de chaque individu. Elle joue notamment un rôle dans le cadre du développement embryonnaire, du maintien des fonctions physiologiques normale, mais est également impliquée dans le développement de plusieurs pathologies. La migration cellulaire repose sur le cytosquelette d'actine. C'est la polymérisation d'actine contre la membrane plasmique qui permet le mouvement des cellules vers l'avant. Cette force ne se traduit par un réel mouvement de la cellule uniquement si des structures d'adhésion engagent physiquement le cytosquelette d'actine à la matrice extracellulaire. À ce jour, différents types de structures d'adhésion ont été décrites. Elles présentent pour la plupart une composition similaire et dépendent d'interactions avec le cytosquelette d'actine. Cependant, nous avons récemment proposé que les structures recouvertes de clathrine puissent également servir de structures d'adhésion.

Les structures recouvertes de clathrine sont principalement connue pour être au cœur de l'endocytose dépendante de la clathrine (EDC). Au cours de l'EDC, les cellules internalisent des portions de membrane et les récepteurs ou cargos qui s'y trouvent. L'EDC participe à la régulation de nombreux récepteur membranaire et est ainsi centrale dans de nombreux processus cellulaire. L'EDC débute par l'accumulation de récepteurs et de leurs ligands dans des invaginations de la membrane plasmique appelés puits recouvert de clathrine. Ces invaginations augment progressivement jusqu'à leur séparation de la membrane ce qui conduit à la formation de vésicules diffusant à l'intérieur de la cellule.

Nous avons donc précédemment montré qu'une sous population de ces PRCs, appelé structures tubulaires de clathrin/AP2 (STCA), peut également servir de structure d'adhésion sur des fibres de collagène. Nous avons d'abord observé que des structures de clathrine s'accumulent le long des fibres de collagène dans des réseaux en 3D. Cette accumulation s'explique par une augmentation locale de la nucléation des structures de clathrine. La nucléation des structure de clathrine étant induite la courbure de la membrane associé à la fibre collagène. Les STAC vont prendre suivre la courbure membranaire et pincer la fibre de collagène. L'accumulation d'intégrines dans ces STAC les transforme en structure d'adhésion que les cellules utilisent pour migrer dans des réseaux en trois dimensions. Mon travail de thèse vient à la suite de ce projet et cherche à faire le lien entre le rôle d'adhésion et le rôle d'endocytose

des structures de clathrine. Plus précisément, j'ai postulé que des ligands liés à la matrice extracellulaire pourraient influencer la dynamique des STCA et ainsi orienter la migration cellulaire.

J'ai d'abord montré que deux ligands connus pour être internalisés par la voie dépendante de la clathrine, le facteur de croissance épidermique (EGF) et les lipoprotéines de basse densité (LDL), sont capables de se lier à des réseaux de collagène. J'ai ensuite mis au point un protocole permettant de transformer ces réseaux décorés en fibres individuelles. Ces fibres sont plus faciles à manier que les réseaux 3D et permettent une meilleure précision dans l'étude de leurs effets sur les cellules. J'ai ensuite déposé des cellules sur un réseau 2D de fibres nues et décoré afin d'observer la distribution des structures de clathrine. J'ai observé que les fibres décorées étaient associées à plus de structures de clathrine que les fibres nues. Cette accumulation est due à une augmentation de la nucléation des structures de clathrine associées aux fibres décorées. Cette nucléation préférentielle dépend notamment du récepteur à l'EGFR mais pas de son activation. J'ai également montré que les cellules produisent plus de protrusions sur les fibres décorées par de l'EGF. Toujours en 2D, j'ai également montré que les cellules appliquent des forces plus importantes à des fibres décorées par rapport à des fibres nues. Ce surplus de forces appliqué aux fibres décorées nécessite les récepteurs des ligands mais également la présence de structure de clathrine. Ces résultats suggèrent que des STCA sont les structures d'adhésion responsables des forces appliquées préférentiellement aux fibres décorées. J'ai ensuite développé un protocole pour créer une interface entre un réseau 3D de collagène nu et un réseau 3D de collagène décoré par de l'EGF. J'ai ainsi pu observer que les cellules migrent préférentiellement en direction du réseau de collagène décoré par de l'EGF. A nouveau, cette migration préférentielle est dépendante à la fois du récepteur à l'EGF mais aussi de la présence de structure de clathrine. Ces résultats révèlent un haptotactisme des cellules pour l'EGF, le fait que les cellules migrent en suivant une molécule liée au substrat. Ces résultats suggèrent également que les structures de clathrine sont les structures d'adhésion à l'œuvre dans cette migration dirigée.

En conclusion, nous proposons un modèle où les cellules peuvent migrer en suivant des ligands liés à la matrice extracellulaire. Dans notre modèle, une distribution asymétrique de ligands entraîne une distribution asymétrique des structures de clathrine à l'intérieur de la cellule. Ces structures adhésives régulent les forces appliquées à la matrice, et donc la distribution asymétrique des forces se conclut par la migration dirigée des cellules. Ce mécanisme permet ainsi aux cellules de suivre des gradients de ligands liés à la matrice et ainsi de s'orienter dans l'organisme.

Titre : Les structures de clathrine dirigent la migration haptotactique en 3D

Mots clés : Haptotactisme, EGF, Clathrine, Migration

Résumé : La migration cellulaire est un processus fondamental au maintien des fonctions physiologiques de l'organisme. Elle est également centrale dans de nombreuses pathologies et entre notamment en jeu lors de la dissémination métastatique. Lorsqu'elles migrent, les cellules utilisent des structures d'adhésion afin de s'appuyer sur leur environnement. Nous avons récemment montré que les puits recouverts de clathrine, plus connus pour leur rôle dans l'endocytose, peuvent également servir de structures d'adhésion. Dans ce manuscrit, je démontre que certains ligands internalisés par la voie d'endocytose clathrine peuvent également se lier à la matrice et orienter la migration cellulaire en régulant les structures adhésives de clathrine.

J'ai commencé par montrer que le collagène est associé à plus de structures de clathrine et a plus de protrusions lorsqu'il est recouvert par des ligands. J'ai ensuite montré que les cellules appliquaient plus de forces sur des fibres de collagènes décorées par des ligands et que ce surplus de force nécessite la présence de structures de clathrine. Enfin j'ai montré que les cellules suivent les ligands liés à des réseaux de collagène en 3D et que cette migration dirigée nécessite également la présence de structures de clathrine. Ce mécanisme de migration pourrait notamment permettre aux cellules de suivre des gradients de ligands liés à la matrice in vivo et ainsi de s'orienter dans l'organisme.

Title: Adhesive clathrin structures support 3D haptotaxis through local force transmission

Keywords: Haptotaxis, EGF, Clathrin, Migration

Abstract: Cell migration is a fundamental process in the development and homeostasis of multicellular organisms. It is also central to many pathologies and it is especially important for metastatic dissemination. When migrating, cells use adhesion structures to push on their substrate in order to move forward. We recently showed that clathrin coated structures, primarily known as endocytic structures, can also serve as adhesion structures. In this manuscript, I show that some ligands internalized through clathrin mediated endocytosis can also bind to the extracellular matrix and orient cell migration using adhesive clathrin structures.

I first showed that ligand-decorated collagen fibers are associated with more clathrin structures and more protrusions. I then showed that cells applied more forces to the ligand-decorated collagen fibers and this extra amount of forces requires the presence of clathrin structures. Finally, I showed that cells can migrate following collagen-bound ligands in 3D, this directed migration also requiring the presence of clathrin structures. Such migration mechanism could be used by cells to follow in vivo gradient of matrix-bound ligands and thus find their way when migrating inside the body.