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**Role of caveolin-3 and caveolae mechanics
in the muscle pathophysiology**

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« Et voilà, on a les droits ! »

François Pignon

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TABLE OF CONTENT

TABLE OF CONTENT	1
LIST OF FIGURES AND TABLE	3
ABBREVIATIONS	4
SUMMARY	7
RESUME	8
1 The skeletal muscle, an essential and highly organized tissue	11
1.2 From the muscle tissue to the single myofiber	11
1.3 The myofiber, a highly specialized architecture	12
1.3.1 The sarcomere and contractility.....	12
1.3.2 The costamere, myofiber integrity and mechanotransduction.....	13
1.3.3 Dysferlin and membrane repair.....	14
1.3.4 Muscle associated signaling pathways.....	15
1.3.4.1 The glucose pathway in the muscle.....	15
1.3.4.2 The IL6/STAT3 pathway in the muscle.....	16
1.4 The specific organelle organization and membrane topology of myofibers	18
1.4.1 Nuclei.....	18
1.4.2 The triad: the sarcoplasmic reticulum and the T-tubule system.....	19
2 Caveolae	21
2.1 Caveolae, specialized plasma membrane invaginations	21
2.2 Caveolae biogenesis	21
2.2.1 Caveolae protein composition	21
2.2.1.1 Caveolins	21
2.2.1.2 Cavins	24
2.2.1.3 Accessory proteins	25
2.2.2 Caveolae, a specific lipid composition.....	25
2.2.3 Caveolae formation, a step by step process	26
2.2.3.1 Minimum requirements	26
2.2.3.2 Caveolin oligomerization and trafficking to the plasma membrane.....	27
2.2.3.3 Cavins recruitment and caveolae invagination	28
2.2.3.4 Recruitment of accessory proteins.....	28
2.3 Caveolae functions	29
2.3.1 Caveolae and cell trafficking.....	29
2.3.2 Caveolae and lipid homeostasis	31
2.3.3 Caveolae and signaling.....	32
2.3.4 Caveolae, membrane tension buffering and mechanoprotection	34
2.4 Caveolae related diseases	35
2.4.1 Lipodystrophy	36
2.4.2 Vascular and pulmonary dysfunction.....	37
2.4.3 Cancer.....	38
2.4.4 Muscle diseases.....	39
3 Cav3 and caveolae in muscle physiology and pathophysiology	42
3.1 Cav3 mutations and muscle diseases	42
3.2 Cav3 and caveolae in muscles	43
3.2.1 Myofiber integrity	43
3.2.1.1 Mechanoprotection	43
3.2.1.2 Membrane repair.....	44
3.2.1.3 Myoblast fusion, muscle atrophy and muscle hypertrophy	45

3.2.2 Myofiber function.....	46
3.2.2.1 Triad formation and function	46
3.2.2.2 Costamere formation.....	47
3.2.2.4 Calcium, NO and muscle contraction	48
3.2.2.4 Glucose metabolism	49
4 Lack of functional caveolae in Cav3 P28L and Cav3 R26Q human dystrophic myotubes results in deficient mechanoprotection and IL6/STAT3 mechanosignaling.....	53
4.1 Objectives and summary	53
4.2 Method	56
4.3 Article.....	64
5 Discussion and perspectives	102
5.1 CAV3 mutations, retention in the Golgi complex and loss of caveolae at the plasma membrane	102
5.2 Caveolae is required for plasma membrane integrity in muscle cells undergoing mechanical stress.....	104
5.3 Caveolin-3 and mechanical stress are negative regulators of the IL6/STAT3 pathway	105
5.4 Caveolin-3 mutations are associated with different clinical phenotypes...	108
REFERENCES.....	112
ANNEX.....	152

LIST OF FIGURES AND TABLE

Figure 1. The structure of the skeletal muscle

Figure 2. The sarcomere

Figure 3. Actin filament sliding allows muscle contraction

Figure 4. The costamere

Figure 5. Model for the mechanism of membrane patching in muscle fibers.

Figure 6. Insulin and exercise mediated GLUT4 translocation at the plasma membrane.

Figure 7. The IL6/STAT3 pathway.

Figure 8. IL6 autocrine functions in skeletal muscle.

Figure 9. Nuclear positioning during myogenic differentiation.

Figure 10. Calcium release and uptake during excitation-contraction coupling controlled by the Triad.

Figure 11. Caveolae observation by electron microscopy.

Figure 12. Caveolin protein family domains and insertion in the plasma membrane.

Figure 13. Cavin structure.

Figure 14. Putative model for caveolar coat assembly and organization.

Figure 15. Fate of Cav1 following caveolar endocytosis.

Figure 16. Caveolae components behavior after caveolae flattening.

Figure 17. Potential role of caveolae in tumor progression.

Figure 18. Muscle disorders associated caveolin-3 mutations.

Figure 19. Model for caveolin dependent dysferlin trafficking.

Figure 20. Caveolin-3 promotes muscle growth.

Figure 21. Modulation of myoblast fusion by caveolin-3.

Figure 22. Synchronization of Cav3 trafficking.

Figure 23. Membrane repair defect in Cav3 P28L and R26Q myotubes.

Figure 24. Cav3 interacts with JAK2.

Table 1. Cav3 mutations and associated caveolinopathies

ABBREVIATIONS

aa : amino acid
AMPK: AMP-activated protein kinase
ATP: adenosine triphosphate
BAR: bin, amphiphysin and rvs
Ca²⁺: Calcium ion
CAF: cancer associated fibroblasts
Cav1, 2, 3: Caveolin-1, -2, -3
CBM: caveolin binding motif
CCP: clathrin coated pits
cdc42: Cell division control protein 42
C/EBP δ : CCAAT-enhancer-binding proteins
CHC: clathrin heavy chain
CK: creatine kinase
CLIC/GEEC: clathrin-independent carriers/GPI-AP enriched early endosomal compartment
COP: coat protein
CRAC: cholesterol recognition/interaction amino acid consensus
CSD: caveolin scaffolding domain
CTx: cholera Toxin
Cys: cystein
DGC: dystrophin glycoprotein complex
DHPR: dihydropyridine receptor
DM: distal myopathy
ECM: extra cellular matrix
EGF: epidermal growth factor
EGFR: epidermal growth factor receptor
EHD2: EH-domain containing 2
EM: electron microscopy
eNOS: endothelial nitric oxide synthase
ER: endoplasmic reticulum
ERK: extracellular signal–regulated kinase
EV: extracellular vesicles
FRET: Förster resonance energy transfer
GD3, GD1a: disialoganglioside 3, 1a
GLUT: glucose transporter
GSL: glycosphingolipids
GM1, GM3: monosialotetrahexosylganglioside 1, 3
gp130: glycoprotein 130

GTP: guanosine triphosphate
HCK: hyperCKemia
HR1/2: helical region 1/2
ICAM-1: intercellular adhesion molecule 1
IF: intermediate filament
IL-6: interleukin-6
IL-6R: IL-6 receptor
IRS-1: insulin receptor substrate 1
JAK1, JAK2: Janus Kinases 1, 2
kDa: kilo Dalton
KO: knock-out
LDL: low density lipoprotein
LGMD 1C: limb girdle muscular dystrophy 1C
LINC: linker of nucleoskeleton and cytoskeleton
MAPK: mitogen-activated protein kinase
MG53: mitsugumin 53
MHC: major histocompatibility complex
MJ: myotendinous junction
mRNA: messenger ribonucleic acid
MT: microtubules
MTOC: microtubule organizing center
MURC: muscle-restricted coiled- coil protein
MVB: multivesicular bodies
NE: nuclear envelop
NFAT: nuclear factor of activated T-cell
NLS: nuclear localization signaling
NMJ: neuromuscular junction
NO: nitrite oxide
OE: overexpression
Paccin2: protein kinase C and casein kinase substrate in neurons protein 2
PCM-1: pericentriolar material protein 1
PEST: sequences enriched in proline, glutamic acid, serine and threonine
Phe: phenylalanine
PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2, or PI(4,5)P2: phosphatidylinositol (4,5)-bisphosphate
PKB/akt: protein kinase B
PKC: protein kinase C
PLA: proximity ligation assay
PM: plasma membrane
PS: phosphatidyleserine
PTRF: polymerase I and transcript release factor
rac1: ras-related C3 botulinum toxin substrate 1

RMD: rippling muscle disease
RUSH: Retention Using Selective Hook
RyR1: ryanodine receptor 1
SDPR: serum deprivation-response protein
Ser: serine
SERCA: sarco-endoplasmic reticulum calcium ATPase pumps
SHP2/PTPN11: Tyrosine-protein phosphatase non-receptor type 11
SOCS: suppressor of cytokine signaling 3
SR: sarcoplasmic reticulum
SRBC: sdr-related gene product that binds to c-kinase
STAT3: signal transducer and activator of transcription 3
TG: triglyceride
TGF- β : transforming growth factor β
Trp: tryptophan
TRPC1: transient receptor potential channel 1
T-tubule: transverse tubule
Tyr: tyrosine
VCAM: vascular cell adhesion molecule
VEGF: vascular endothelial growth factor
VIP-21: vesicular integral protein 1
WT: wild type

SUMMARY

Caveolae are plasma membrane invaginations that require caveolin proteins for their biogenesis. Recently, our laboratory reported a new role for caveolae in the cell response to mechanical stress (Sinha et al, Cell, 2011). Mutations in the CAV3 gene (muscle isoform), which lead to Cav3 retention in the Golgi complex, are associated with muscle disorders. My project consists in identifying the functional link between CAV3 mutations and muscle disorders, which exhibit defects in membrane integrity and repair, and in muscle homeostasis. Giving the rising interest on caveolae in the cell mechanical response, we focused on their specific role in human CAV3 related muscle diseases. To do this, we studied human myotubes from healthy patients, or bearing the CAV3 P28L or R26Q mutation, allowing the study of endogenously expressed CAV3 mutations. First, we showed a drastic decrease of caveolae structures at the plasma membrane of CAV3 mutant myotubes due to Cav3 mutant retention in the Golgi complex. As a consequence, we could show that mutant myotubes were not capable of buffering the increase of membrane tension upon mechanical stress, resulting in failed mechanoprotection. Considering the possible role of caveolae mechanics in signaling (Nassey & Lamaze, 2012), we next wanted to know whether the interleukin-6 (IL6) signaling pathway could be differently regulated in mutant and wild type myotubes under rest and upon mechanical stress, as it is one of the major signaling pathways in muscle cells, and is tightly linked to muscle contraction. We found that Cav3 could negatively regulate the IL6 signaling pathway. Furthermore, IL6 signaling pathway is also negatively regulated by mechanical stress in a Cav3-dependent manner. As a result, hyperactivation of the IL6 pathway, and failed mechano-regulation of the pathway were observed in mutant myotubes. Interestingly, mutated myotubes phenocopy Cav3 depletion, and the phenotype is reversible with caveolae reformation upon re-expression of the WT form of Cav3. This confirms the direct link between CAV3 mutations, the absence of caveolae and the observed phenotype.

RESUME

Les cavéoles sont des invaginations de la membrane plasmique dont la biogénèse nécessite la présence d'une famille de protéines appelées cavéolines. Récemment, mon laboratoire d'accueil a décrit un nouveau rôle pour les cavéoles dans la réponse des cellules à des stress mécaniques (Sinha et al., 2011). Des mutations dans le gène CAV3 (isoforme spécifique du muscle), qui ont pour conséquence la rétention de la protéine Caveoline-3 (Cav3) dans l'appareil de Golgi, ont été décrites comme associées à des pathologies musculaires. Mon projet consiste en l'identification du lien fonctionnel entre ces mutations de CAV3 et les pathologies musculaires associées qui ont pour caractéristiques un défaut d'intégrité et de réparation membranaire, ainsi que des dérégulations dans l'homéostasie du muscle. Le rôle des cavéoles dans la réponse mécanique de la cellule suscitant un intérêt grandissant, nous nous sommes plus particulièrement intéressé à cet aspect dans le contexte des mutations de CAV3 associées à des pathologies musculaires. Pour ce faire, nous avons étudié des myotubes humains immortalisés à partir de biopsies de patients sains, ou portant les mutations CAV3 P28L ou CAV3 R26Q, ce qui nous a permis d'étudier ces mutations à un niveau d'expression endogène.

Dans un premier temps, nous avons montré une diminution importante de structures caveolaires à la membrane plasmique des myotubes mutés pour Cav3, ce qui résulte de la rétention du mutant de Cav3 dans l'appareil de Golgi. En conséquence, nous avons montré que les myotubes mutants n'étaient plus capable de tamponner l'augmentation de la tension de membrane lorsqu'ils sont soumis à un stress mécanique, ce qui conduit à un défaut de mécanoprotection de ces myotubes.

En considérant le potentiel rôle de la mécanique des cavéoles dans la signalisation cellulaire (Nassey & Lamaze, 2012), nous avons ensuite voulu savoir si la voie de l'interleukine-6 (IL6) pouvait être régulée différemment dans les myotubes sains et les myotubes mutants lorsqu'ils sont au repos ou soumis à un stress mécanique. En effet, la

voie IL6 est une des voies de signalisation majeures dans le muscle et est étroitement associée avec la contraction musculaire. Nous avons pu montrer que Cav3 régule négativement la voie IL6, et celle-ci est également inhibée lorsque les myotubes sont soumis à un stress mécanique et ce, dépendamment de Cav3. Cela a pour conséquence une hyperactivation de la voie IL6 au repos, ainsi qu'un défaut de mécanorégulation de la voie IL6 dans les myotubes mutés soumis à un stress mécanique. De manière intéressante, nous avons pu montrer que les myotubes mutants avaient le même phénotype que des myotubes dans lesquelles Cav3 a été déplétée. De plus, le phénotype observé chez les mutants peut être inversé lorsque l'on reforme des cavéoles à la membrane après réexpression de la forme sauvage de Cav3. Ceci confirme donc un lien direct entre les mutations de CAV3, l'absence de cavéoles à la membrane plasmique et le phénotype observé dans les mutants.

- INTRODUCTION -

1 The skeletal muscle, an essential and highly organized tissue

Several types of muscle tissues exist within the organism: cardiac muscle, smooth muscle and skeletal muscle. In the context of my PhD work, I will focus on the most represented one in our body, the skeletal muscle.

1.1 The skeletal muscle, a central role in the organism

In human, skeletal muscles represent approximately 40% of total body weight and concentrates 50-70% of total proteins. It is composed of 20% proteins, 70% of water, and the rest consists in carbohydrates, mineral fat and inorganic salts (Frontera & Ochala, 2014). Skeletal muscles, as the name indicates, are linked to the skeleton, through tendons attached to the bones, and have two main functions in the body. One is to regulate glucose and lipid homeostasis, and the second is to provide support and movements through force generation initiated by muscle contractility.

In this chapter, the particular organization and composition of the skeletal muscle will be detailed, highlighting how the fine structure of this organ enables muscle functions.

1.2 From the muscle tissue to the single myofiber

The muscle tissue is composed of satellite cells, myofibers, connective tissue, adipocytes and motoneurons fibers. Satellite cells, also called myoblasts, differentiate and fuse into myotubes, which undergo further maturation process. The fully differentiated muscle cells are myofibers, which are multinucleated cells that can reach several cm long with an average diameter of 100 μ m (Bentzinger et al., 2012). Myofibers are surrounded by connective tissue called the epimysium and assemble in bundles surrounded by the

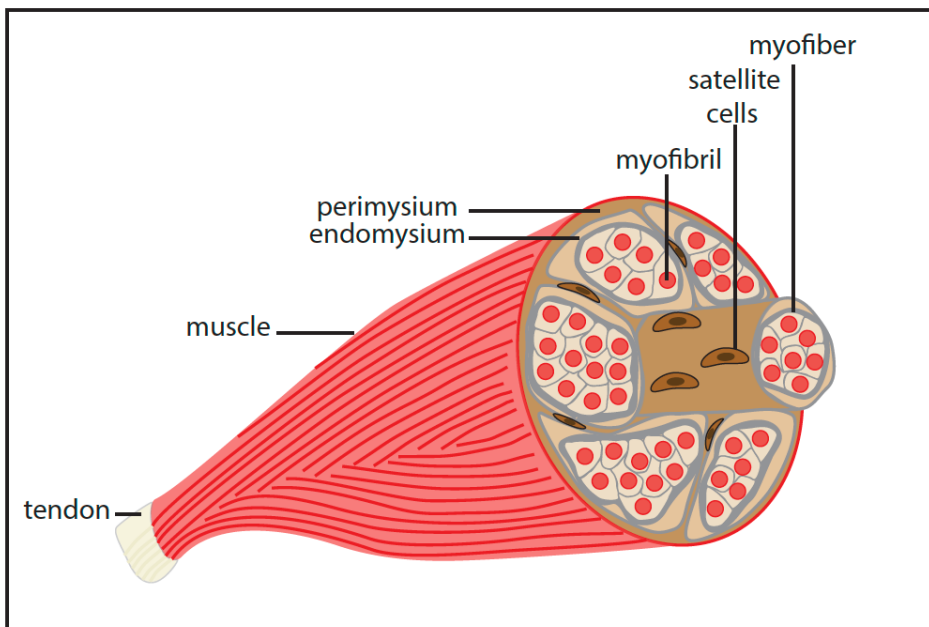


Figure 1. The structure of the skeletal muscle

In the skeletal muscle, satellite cells differentiate and fuse together to form the single unit of the muscle tissue: the myofiber. Myofiber contains a large number of myofibrils, which play a key role in contraction. Myofibers are surrounded by connective tissues called endomysium, assembled in bundles, themselves surrounded by the perimysium. Based on (Frontera & Ochala, 2014).

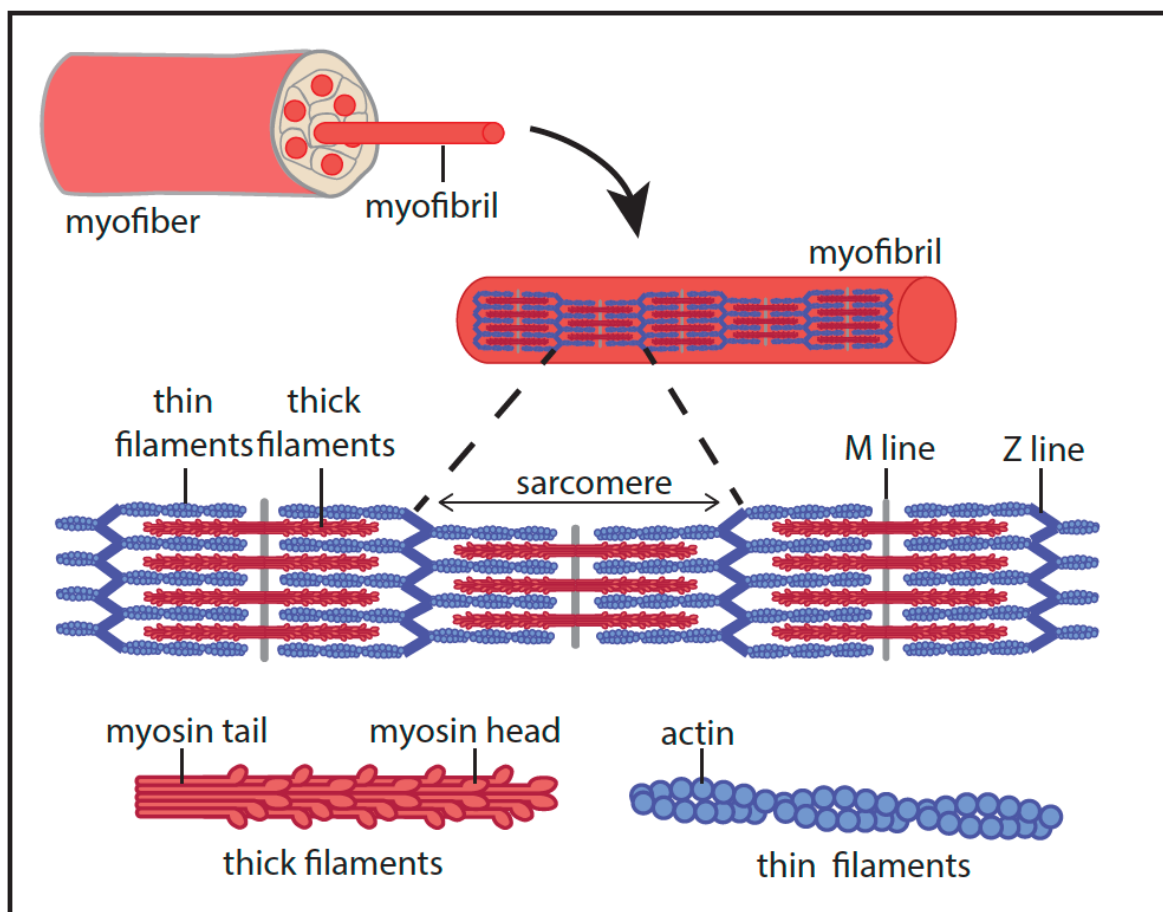


Figure 2. The sarcomere

The sarcomere is the structural unit of a myofibril, allowing contraction. Sarcomere formation relies on the association between thick filaments made of myosin and thin filaments made of actin. Thick filaments are docked on both sides of the M line at the center of the sarcomere. They are interdigitated with thin filaments, which are docked on the Z line at each extremity of the sarcomere. Based on (Frontera & Ochala, 2014).

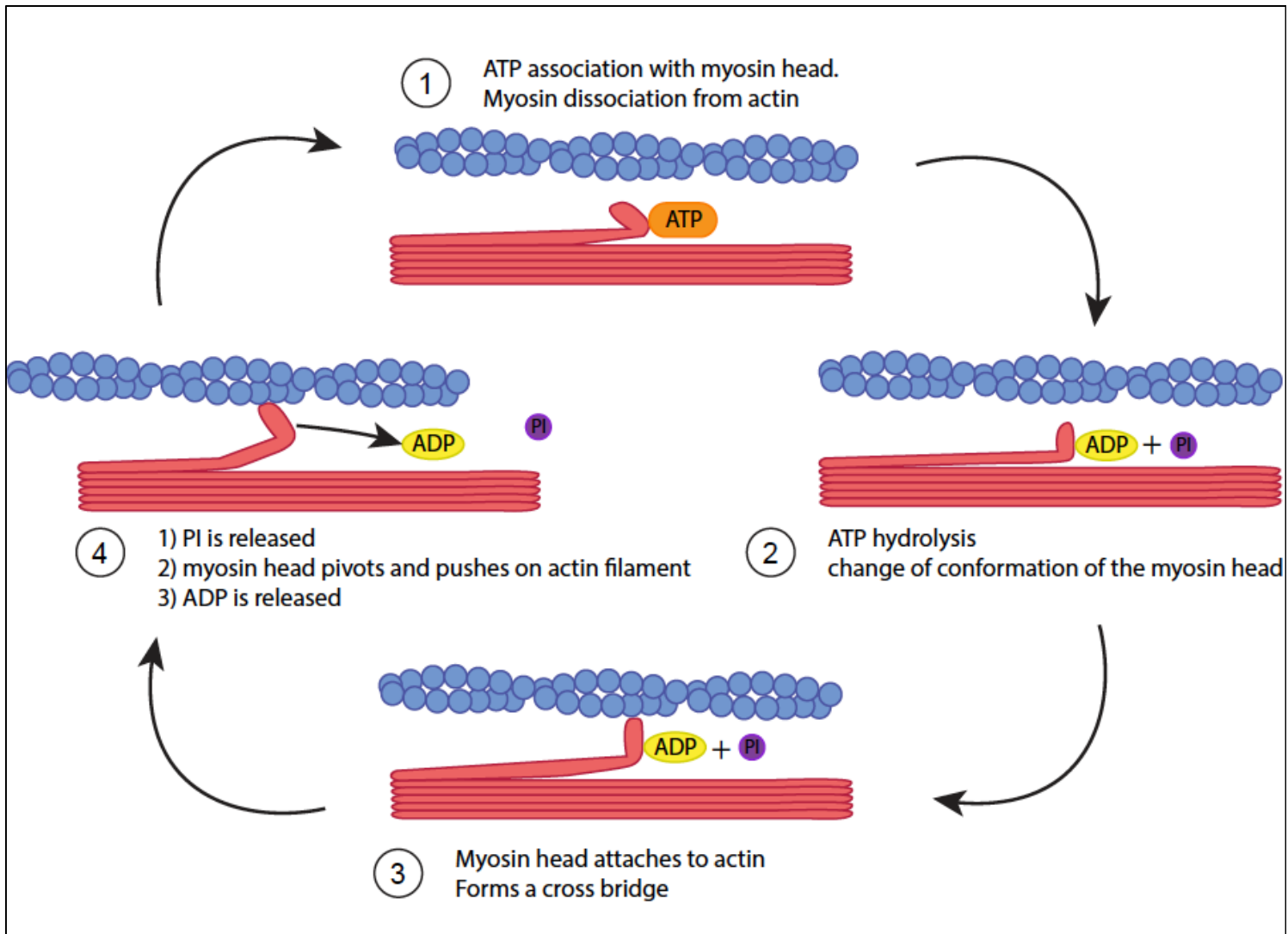


Figure 3. Actin filament sliding allows muscle contraction

1- ATP binds to myosin head. 2- Myosin ATPase activity triggers ATP hydrolysis. ADP + PI remain bound to myosin and induce conformational change of its head. 3- In this conformation, the myosin head binds to actin and forms a cross bridge. 4- The release of PI leads to the rotation of myosin head which pushes the actin filament. Finally, the release of ADP allows new ATP molecule binding and makes the cycle start again. Based on (Frontera & Ochala, 2014).

perimysium (Frontera & Ochala, 2014). Satellite cells are located between the myofiber's plasma membrane (PM), called sarcolemma, and the basal lamina (**Figure 1**). They can self-renew and are key for muscle regeneration (Collins et al., 2005).

The myofiber, the single unit of the muscle, is highly specialized and has a particular protein and organelle content, as well as a specific sarcolemma topology further detailed in the next chapters.

1.3 The myofiber, a highly specialized architecture

1.3.1 The sarcomere and contractility

Each myofiber contains multiple myofibrils, themselves composed of an assembly of sarcomeres. A sarcomere is the muscle contractile unit consisting of a specific arrangement of myofilaments. Myofilaments can be of two types: they can be thick filaments composed of myosin and thin filaments made of actin and troponin. Thin actin filaments are docked on one side to a Z line, mainly composed of α -actinin. One Z line can be found at each extremity of a sarcomere. These actin filaments, emanating symmetrically from each Z line are connected and interdigitate with thick myosin filaments that compose the H zone. Myosin filaments are docked on both sides of a central M line (**Figure 2**). The contraction of skeletal muscle relies on myosin and actin interaction, that form cross-bridge through myosin heads binding to actin (Huxley, 1969), and is dependent on calcium release (Szent-Györgyi, 1975). This is followed by the sliding of actin filaments, provoked by myosin heads movement in an ATP (adenosine triphosphate) dependent manner (Holmes & Geeves, 2000; Huxley, 1969; Huxley, 1957). ATP is provided by the mitochondria network or by glycogenolysis. This mechanism is detailed in **Figure 3**.

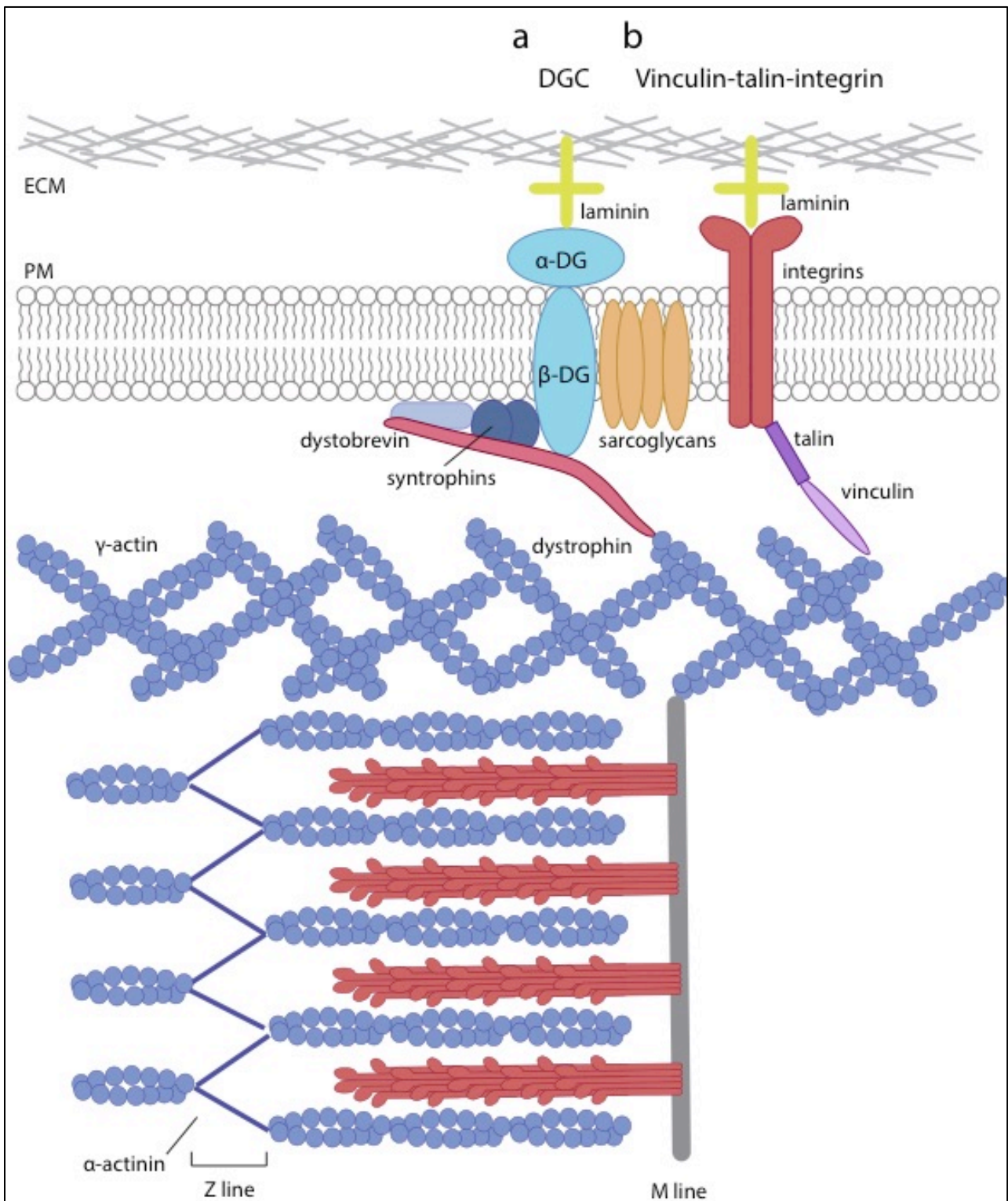


Figure 4. The costamere

Costameres are composed of two protein complexes, the dystrophin glycoprotein complex (DGC) and the vinculin-talin-integrin complex. They are located at the plasma membrane of myofibers and aligned with Z and M lines of sarcomeres.

a) The DGC contains dystrophin, the scaffold protein linking the actin cytoskeleton to the plasma membrane and the extracellular matrix (ECM). Dystrophin bridges γ -actin through its N-terminal domain, to β -dystroglycan through its C-terminal domain. β -dystroglycan is a transmembrane protein that forms a complex with the α -dystroglycan binding directly to laminin in the ECM. In addition, dystrophin also interacts with dystobrevin and syntrophins, both cytosolic proteins associated with the plasma membrane. The sarcoglycans, another set of proteins, stabilizes this complex. Based on (Cohn & Campbell, 2000). b) Vinculin interacts with the extremity of actin bundles linked to sarcomeres, on one side, and with talin on the other. Talin is the scaffold protein linking vinculin to integrins, transmembrane proteins that bind laminin in the ECM. Based on (Sabatelli et al., 2012).

Other actors are involved in the filament stabilization, such as troponin, tropomyosin, titin, nebulin or desmin (the role of desmin is further detailed in the next chapter) (Au, 2004).

1.3.2 The costamere, myofiber integrity and mechanotransduction

Costameres are protein complexes located at the sarcolemma, which are arranged perpendicularly to myofilaments, at a specific distance and are mainly aligned with M lines, but also with Z lines (Pardo et al., 1983). They allow sarcomeres to anchor to the sarcolemma, but also to link the sarcolemma, the sarcomeres, and the cortical actin to the extracellular matrix. The costameres are mainly composed of two protein complexes, the dystrophin-glycoprotein complex (DGC) and the vinculin-talin-integrin complex.

The DGC is the protein complex that allows to link the actin-based cortical cytoskeleton to laminin in the extracellular matrix (ECM), and is crucial for sarcolemma integrity (Yoshida & Ozawa, 1990). It contains both cytoplasmic and transmembrane proteins such as dystrophin, sarcoglycans, dystroglycan, dystrobrevins and syntrophins (**Figure 4a**). Dystrophin is the scaffold protein, binding γ -actin through its N-terminal domain (Ervasti & Campbell, 1993), a transmembrane protein, the β -dystroglycan its carboxyl-terminus, and cytoplasmic proteins such as dystrobrevins and syntrophins. Dystroglycans consist in a heterodimer of two dystroglycan isoforms. β -dystroglycan is a transmembrane protein that binds dystrophin on its C-terminal part, and its extracellular partner α -dystroglycan that binds laminin in the extracellular matrix (Ervasti & Campbell, 1993). The precise role of sarcoglycans is not yet eluded, but they definitely contribute to the stabilization of the DGC complex, as deficient mice show loss of sarcolemmal integrity (Duclos et al., 1998). Finally, syntrophin and dystrobrevin complete this complex to supply an additional layer of dystrophin anchorage to the sarcolemma (Lapidos et al., 2004).

The vinculin-talin-integrin complex allows a link between the sarcomeres, the sarcolemma and the ECM (**Figure 4b**). Vinculin is a membrane associated cytoplasmic protein that

was initially found to bind the extremity of actin filament bundles (Geiger et al., 1980; Hüttelmaiere et al., 1997). Vinculin interacts with talin, a scaffold protein that can also bind β -integrin and actin (BurrIDGE & Mangeat, 1984; Calderwood et al., 2002; Lee et al., 2004). Furthermore, interaction between talin and β -integrin induces β -integrin activation and further binding to collagen or laminin in the ECM (Calderwood et al., 2002), which allows mechanotransduction (De Palma et al., 2013).

A few more proteins are involved in the organization and stability of sarcomeres, in particular desmin and the clathrin heavy chain (CHC). Desmin is an intermediate filament (IF) that is essential for muscle architecture since it connects sarcomeres with the sarcolemma, nuclei and other organelles (Paulin & Li, 2004). Desmin filaments are cross-linked by plectins, create a network and bind to integrins (Steinböck & Wiche, 1999). Recently, an unconventional role for CHC usually associated with endocytosis was described in muscle. At the sarcolemma, CHC was shown to be organized as large plaques that recruit α -actinin for proper anchorage and organization of sarcomere (Vassilopoulos et al., 2014).

Costameres are thus essential for sarcomere anchorage and stability, myofiber integrity and mechanotransduction. No surprisingly, mutations in most of costamere proteins are associated with different types of myopathies (reviewed in Jaka et al., 2015).

1.3.3 Dysferlin and membrane repair

Dysferlin is a transmembrane protein that is key for membrane repair in muscle (Bansal et al., 2003). It localizes at the sarcolemma or near T-tubules (transverse-tubule) (further detailed in chapter 1.4.2) and can be recruited on site of membrane injury. Upon membrane damage, dysferlin is recruited in a Ca^{2+} -dependent manner and, together with MG53 (mitsugumin 53) and caveolin-3 (Cav3), will induce fusion of intracellular vesicles, and injured sarcolemma in order to reseal the wound (Cai et al., 2009; Lennon et al., 2003). The role of Caveolin-3 in this process will be further detailed in *chapter 3.1.2.4*.

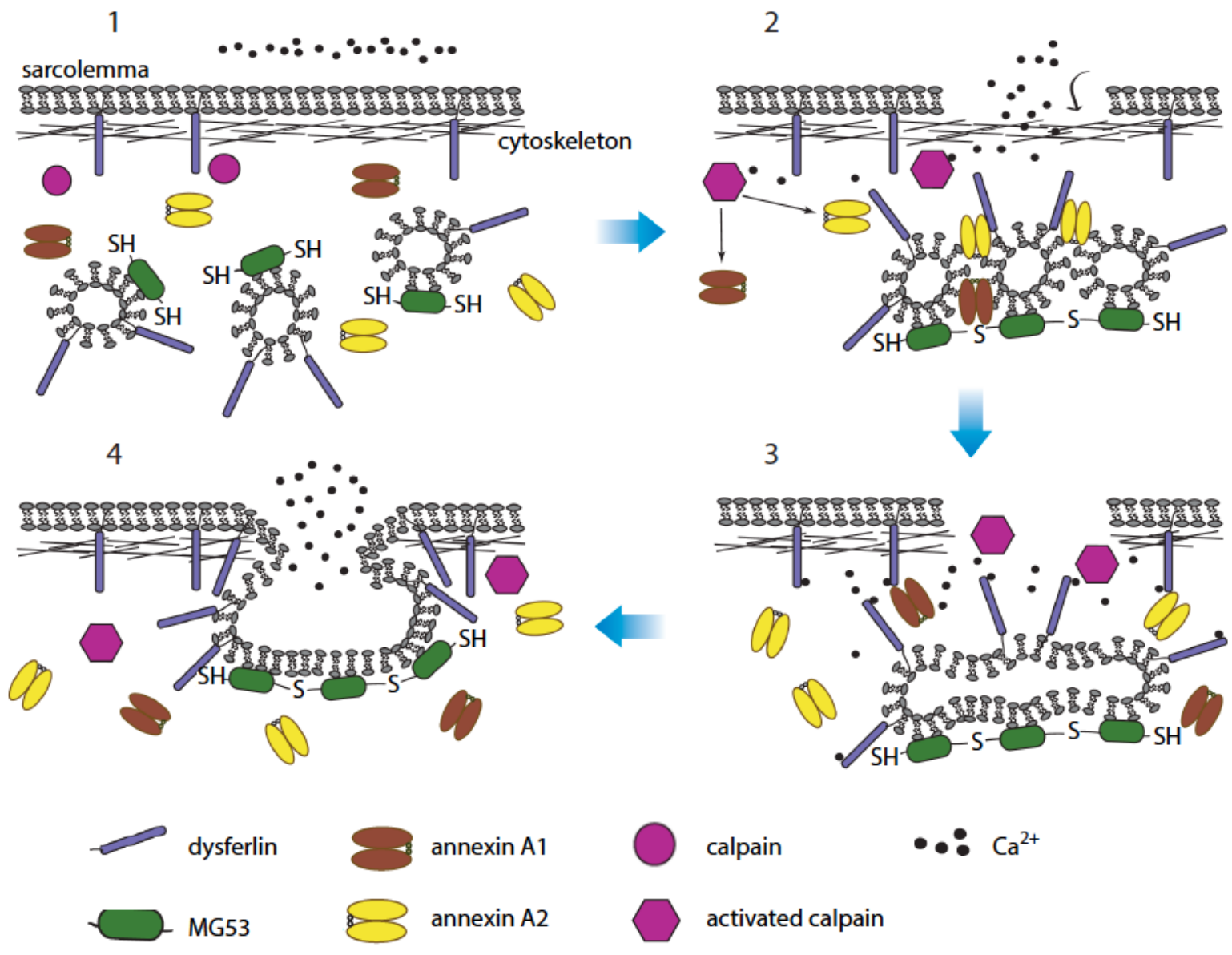


Figure 5. Model for the mechanism of membrane patching in muscle fibers.

1) In the intact muscle fiber, Mitsugumin 53 (MG53) is on sarcoplasmic vesicles in a reduced form, and the concentration of calcium in sarcoplasm is lower than in the extracellular matrix.

2) Upon membrane damage, exposure of the intracellular environment to oxidative agents leads to oxidation and oligomerization of MG53 proteins on sarcoplasmic vesicles. Increased calcium concentration around the injury site also activates calpain. Activated calpain may cleave annexin proteins, which subsequently mediate accumulation of vesicles near the site of damage.

3) Dysferlin senses the calcium that floods into the fiber and triggers fusion of accumulated vesicles. Activated calpain also degrades the cytoskeleton near the damaged area, making easier the fusion of vesicles with the plasma membrane.

4) Vesicles fuse with the sarcolemma and form a patch that seals the membrane. Abbreviation: SH, reduced sulfur moieties in MG53. Adapted from (Tabebordbar, Wang, & Wagers, 2013).

Dysferlin can also be associated with intracellular vesicles and upon membrane damage, it is proposed that these vesicles could fuse with lysosomes to form larger vesicles ready to be recruited for membrane resealing (McDade & Michele, 2014). Dysferlin-dependent membrane repair in muscle is summarized in **Figure 5**.

1.3.4 Muscle associated signaling pathways

Many signaling pathways have been shown to be essential for muscle integrity and functions. In this chapter, I chose to focus on the glucose pathway that is tightly regulated in muscle tissue to maintain glucose and lipid homeostasis, and the IL6 pathway, which is important for muscle tissue integrity and homeostasis (reviewed in Muñoz-Cánoves et al., 2013).

1.3.4.1 The glucose pathway in the muscle

The glucose pathway in skeletal muscle is very important for homeostasis of the muscle and the whole organism. It has been observed very early that glucose was uptaken in skeletal muscle during exercise. Glucose can be transported in cells through specific transporters of the GLUT (glucose transporter) family, and more specifically GLUT1 and GLUT4 in skeletal muscle (Devaskar & Mueckler, 1992). GLUT1 being expressed at very low levels, GLUT4 is the major glucose transporter in muscle cells (Gaster et al., 2000; Zisman et al., 2000). In resting condition, GLUT4 is stored in large endosomal structures, which formation requires the specific clathrin isoform CHC22 (clathrin heavy chain 22), and that can be recruited upon insulin stimulation or muscle contraction (Karlsson et al., 2009; Lauritzen et al., 2010; Ploug et al., 1998; Vassilopoulos et al., 2009). These two stimuli have been proposed to recruit different pools of GLUT4 containing vesicles, as an additive effect was observed for their recruitment when muscle fibers are stimulated with both insulin and muscle contraction (Ploug et al., 1998). Once in the cell, glucose can

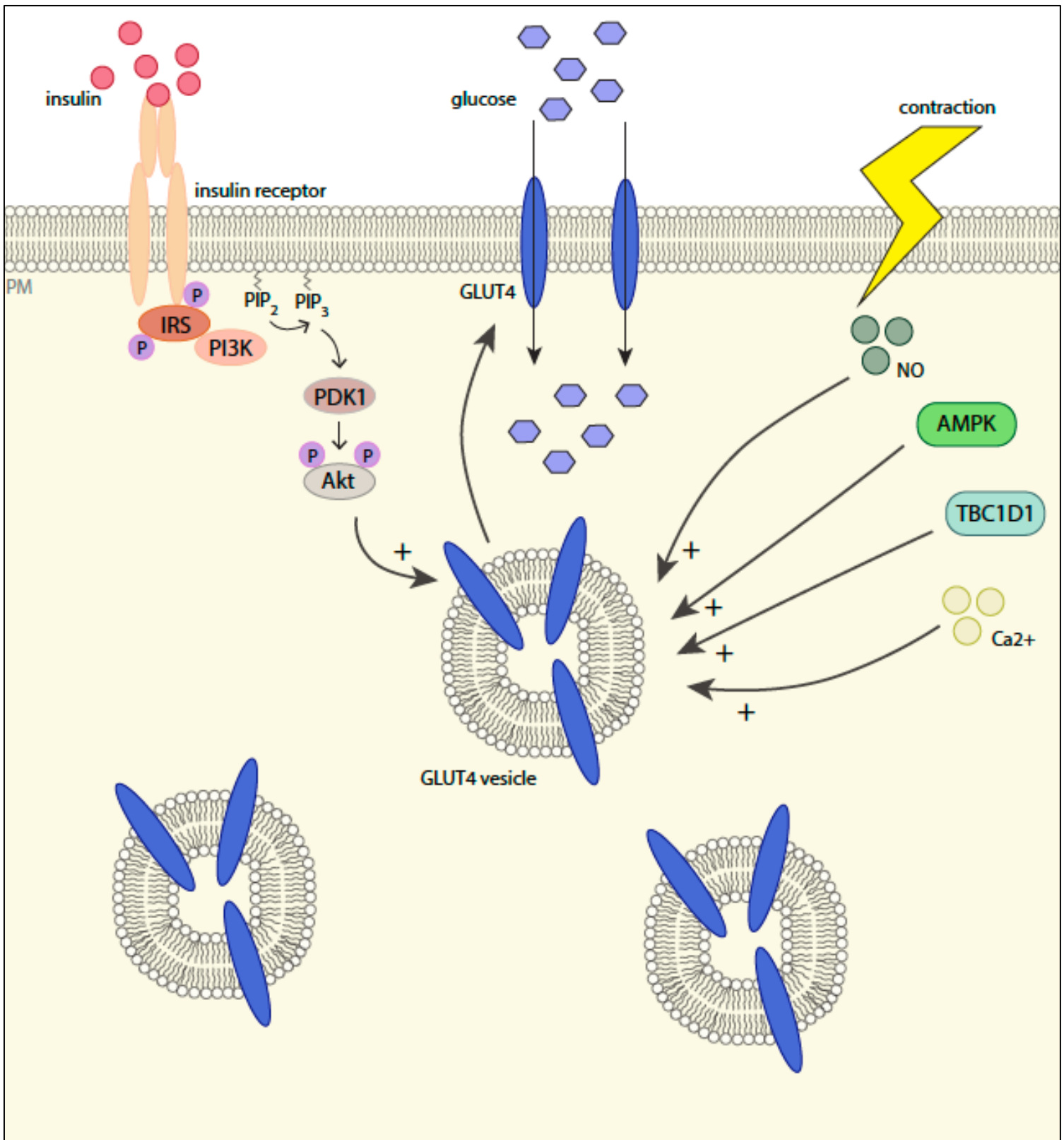


Figure 6. Insulin and exercise mediated GLUT4 translocation at the plasma membrane.

Upon insulin binding, the insulin receptor gets phosphorylated, triggering the phosphorylation of the insulin receptor substrate (IRS) that recruits and activates the Phosphoinositide 3-Kinase (PI3K). This kinase converts PIP₂ to PIP₃, a docking platform for the recruitment of PDK1 and Akt. Once activated, Akt promotes the recruitment of GLUT4 containing vesicles at the plasma membrane, leading to glucose entry in the cell. Based on (Leto & Saltiel, 2012).

Muscle contraction can have many cellular consequences. Among them, Ca²⁺ release increases NO, AMPK and TBC1D1 activities, all promoting the recruitment of GLUT4 vesicles at the plasma membrane. Based on (Richter & Hargreaves, 2013).

undergo glycolysis to provide the ATP needed for muscle contraction or glycogenesis for muscle glycogen storage (Hansen et al., 1995). Then, the stored glycogen will be used for rapid ATP production during the next exercise, or will follow glycogenolysis pathway to provide glucose in fasting diet for example. Glucose uptake mechanism is summarized in **Figure 6**.

1.3.4.2 The IL6/STAT3 pathway in the muscle

Interleukin-6 (IL6) is one of the major cytokine produced by the muscle and plays autocrine, paracrine and endocrine roles (Muñoz-Cánoves et al., 2013; Pedersen & Febbraio, 2008). IL6 is produced and secreted during exercise, and thus during muscle contraction (Steensberg et al., 2002). Several pathways can trigger IL6 production, all dependent on contraction. It was proposed that nitric oxide (NO), produced during exercise, could induce IL6 mRNA expression (Steensberg et al., 2007). NO also increases IL6 protein expression through the MAPK (Mitogen activated kinase) pathway (Makris et al., 2010). Increased calcium levels observed during contraction might also regulate IL6 mRNA (messenger ribonucleic acid) expression. Indeed calcium binding to calcineurin leads to the dephosphorylation of nuclear factor of activated T-cell (NFAT). Then, NFAT translocates to the nucleus where it controls, among others, the IL6 mRNA transcription (Crabtree, 2001; Keller et al., 2006). The decrease of glycogen concentration, induced by glycogenolysis, are linked to the phosphorylation of P38 MAPK (mitogen-activated protein kinase) that promotes IL6 mRNA expression (Chan et al., 2004). Finally, IL6 itself can trigger its own expression probably via activation of AMP-activated protein kinase (AMPK) although this mechanism is not fully elucidated (Keller et al., 2003; Weigert et al., 2007).

IL6 signal transduction depends on its binding to the glycoprotein 130 (gp130), a ubiquitously expressed transmembrane receptor, and to the IL6 receptor (IL6R). IL6 binds first to the IL6R, that can be either at the plasma membrane or soluble in the extracellular

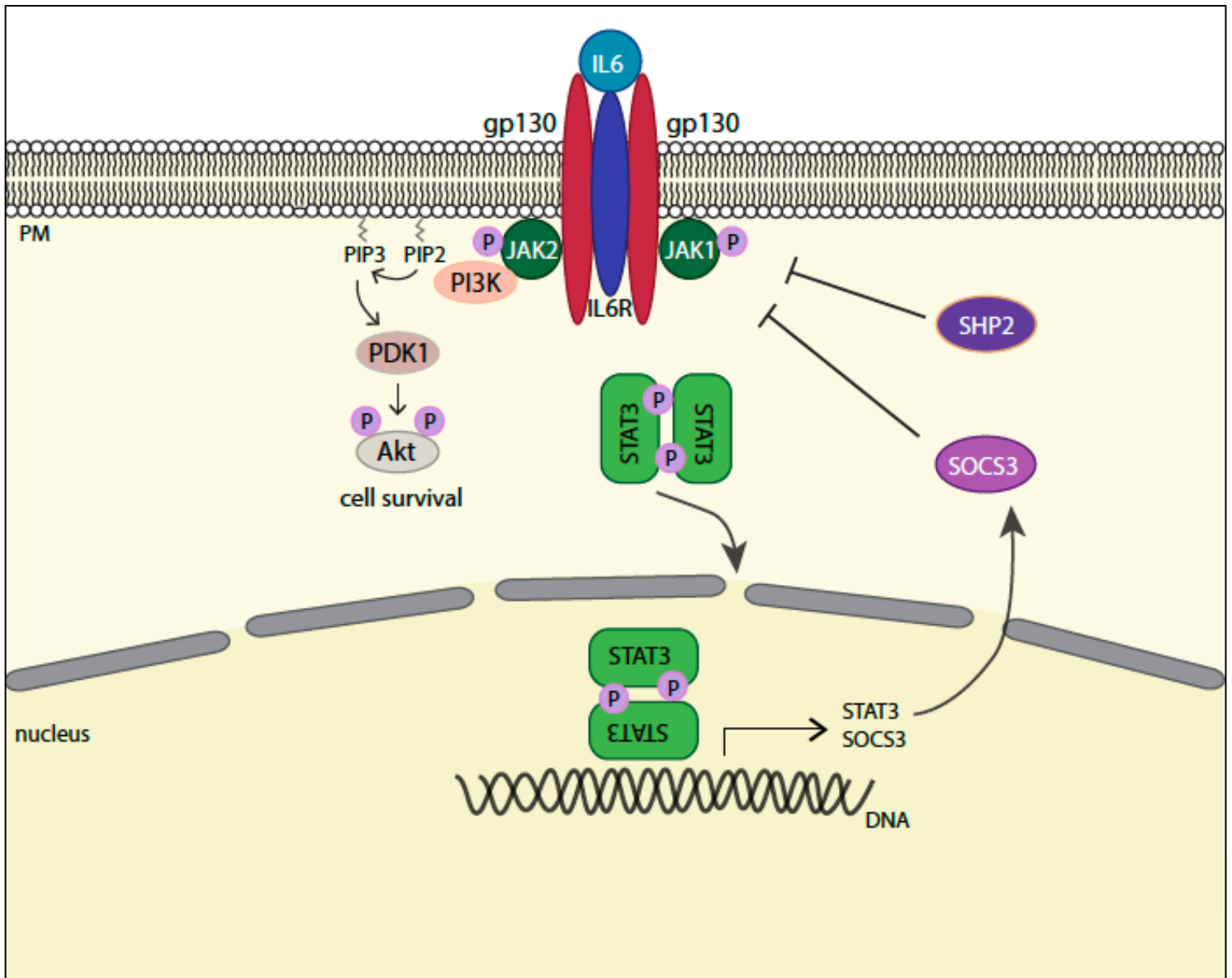


Figure 7. The IL6/STAT3 pathway.

IL6 first binds to the IL6 receptor (IL6R). Then, the complex associates with two gp130 sub-units. JAK1 and JAK2 are then recruited and activated by phosphorylation, leading to the recruitment of the PI3K. PI3K converts PIP₂ to PIP₃, a docking platform for the recruitment of PDK1 that phosphorylates Akt. Finally, Akt activation promotes cell survival. The activation of JAKs also leads to STAT3 phosphorylation, followed by its dimerization and nuclear translocation. In the nucleus, it acts as a transcription factor and regulates the expression of many genes, including STAT3 itself but also SOCS3. SOCS3, together with SHP2, are negative inhibitors of the pathway and therefore key are for the termination of the signal. Based on (Heinrich et al., 2003).

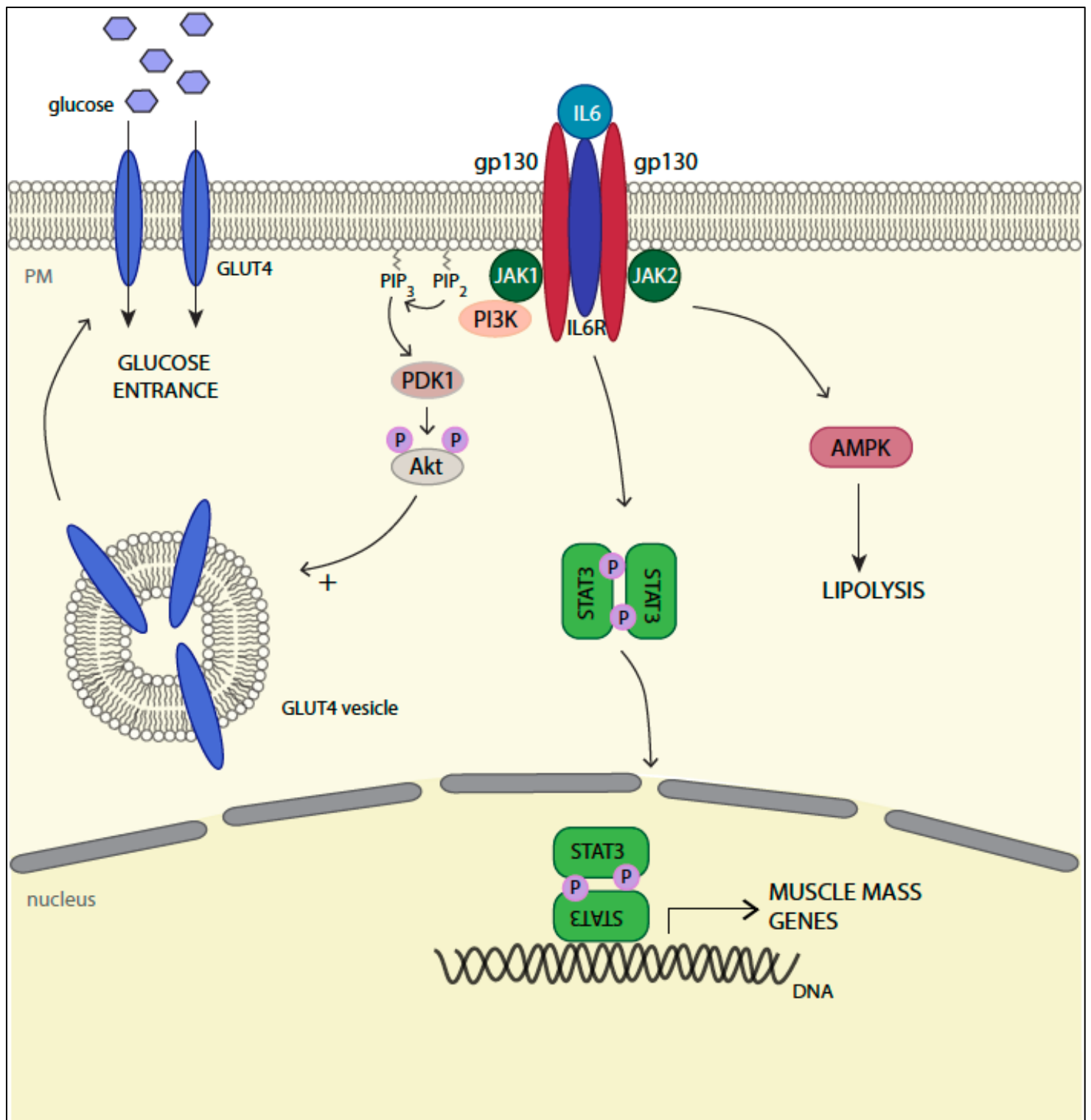


Figure 8. IL6 autocrine functions in skeletal muscle.

In skeletal muscle, the IL6 pathway is associated with glucose and lipid homeostasis. IL6 signaling activation can participate to glucose entry through Akt-dependent GLUT4 vesicles translocation at the plasma membrane. It is also involved in lipolysis through AMPK activation. IL6 activated-STAT3 controls the transcription of several genes such as *FBXO32* or *C/EBP δ* , essential for the control of muscle mass.

environment, and then to gp130, leading to initiation of the signal (Heinrich et al., 2003). Janus Kinases 1 and 2 (JAK1 and JAK2) are recruited to the plasma membrane, and associate to the intracellular tail of gp130 to be activated (Babon et al., 2014; Haan et al., 2001; Haan et al., 2000). Cytosolic STAT3 (signal transducer and activator of transcription 3) is then recruited to the complex and undergoes phosphorylation and dimerization, both required for its subsequent nuclear translocation and activation of STAT related gene transcription (Milocco et al., 1999). JAK activation can also activate AMPK and the subsequent PI3K/Akt (PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase PKB/Akt: protein kinase B) pathway (Heinrich et al., 2003). Two different ways to terminate the signal have been described, and rely on SOCS3 (suppressor of cytokine signaling 3) and SHP2/PTPN11 (Tyrosine-protein phosphatase non-receptor type 11) proteins (Heinrich et al., 2003). The IL6/JAK/STAT pathway in muscle is summarized in **Figure 7**.

The IL6/STAT3 pathway in muscle tissue has been shown to be important for satellite cell activation during muscle regeneration, and its overactivation can cause satellite cell exhaustion (Price et al., 2014; Tierney et al., 2014; Toth et al., 2011). Interestingly, it seems that the main IL6 source involved in muscle regeneration is secreted from infiltrated macrophages and neutrophils (Zhang et al., 2013).

In myotubes, the IL6 pathway contributes to homeostasis, as it enhances lipolysis and glucose uptake by triggering GLUT4 translocation through the activation of the AMPK and PI3K pathways (see chapter 1.3.3.1) (Carey et al., 2006; Wolsk et al., 2010). STAT3 activity is mostly associated with muscle mass. Indeed, in extreme conditions such as high dose and long term IL6 exposure or IL6 overexpression, increased muscle proteolysis with decreased muscle mass have been observed (Ebisui et al., 1995; Franckhauser et al., 2008). This explains the description of IL6/STAT3 induced muscle wasting phenotype in the context of cancer cachexia (Bonetto et al., 2011). Autocrine functions of the IL6 pathway in skeletal muscle are summarized in **Figure 8**.

1.4 The specific organelle organization and membrane topology of myofibers

In myofibers, the organelle organization and distribution is adapted to the very particular elongated shape of these multinucleated cells, highly enriched with myofibrils. I will focus here on two main organelles that are highly adapted to muscle function: the nuclei and the sarcoplasmic reticulum. The sarcoplasmic reticulum (SR) will be described together with the T-tubule system, as they form a particular structure called the triad.

1.4.1 Nuclei

Nuclei are located at the periphery of the myofibers, right below the sarcolemma, in a regular spaced manner (Bruusgaard, 2006). This specific localization requires nuclear movements occurring through sequential steps (Roman & Gomes, 2017). The first step, nuclear centration is dependent on microtubules (MT) that allow the gathering in the center of the cell of newly integrated nuclei when myoblasts fuse with a myotube. In myotubes, the microtubule organizing center (MTOC) has the particularity to be located on the nuclear envelop (NE) instead of the centrosomes as classically observed in other cell types, leading to MT nucleation from the nuclei (Tassin et al., 1985). In addition to MT, other important players are involved in this process, such as the dynein/dynactin motor and cdc42, Par3 and Par6 proteins (Cadot et al., 2012). Nuclei then align and spread along the longitudinal axis. This process is dependent on the LINC (linker of nucleoskeleton and cytoskeleton) complex, and more particularly on nesprin-1 α , that recruits centrosomal proteins such as PCM-1 (pericentriolar material protein 1) and Akap450. PCM-1 allows the recruitment of motors such as dynein/dynactin and kinesins, although kinesin-1 can be recruited by nesprin-1 itself, leading to nuclear movement (Espigat-Georger et al., 2016; Wilson & Holzbaur, 2015). Akap450 regulates MT nucleation and is crucial for nuclear positioning (Gimpel et al., 2017). Finally, nuclei move towards the periphery of the cell where they are anchored. Once myofibrils are formed,

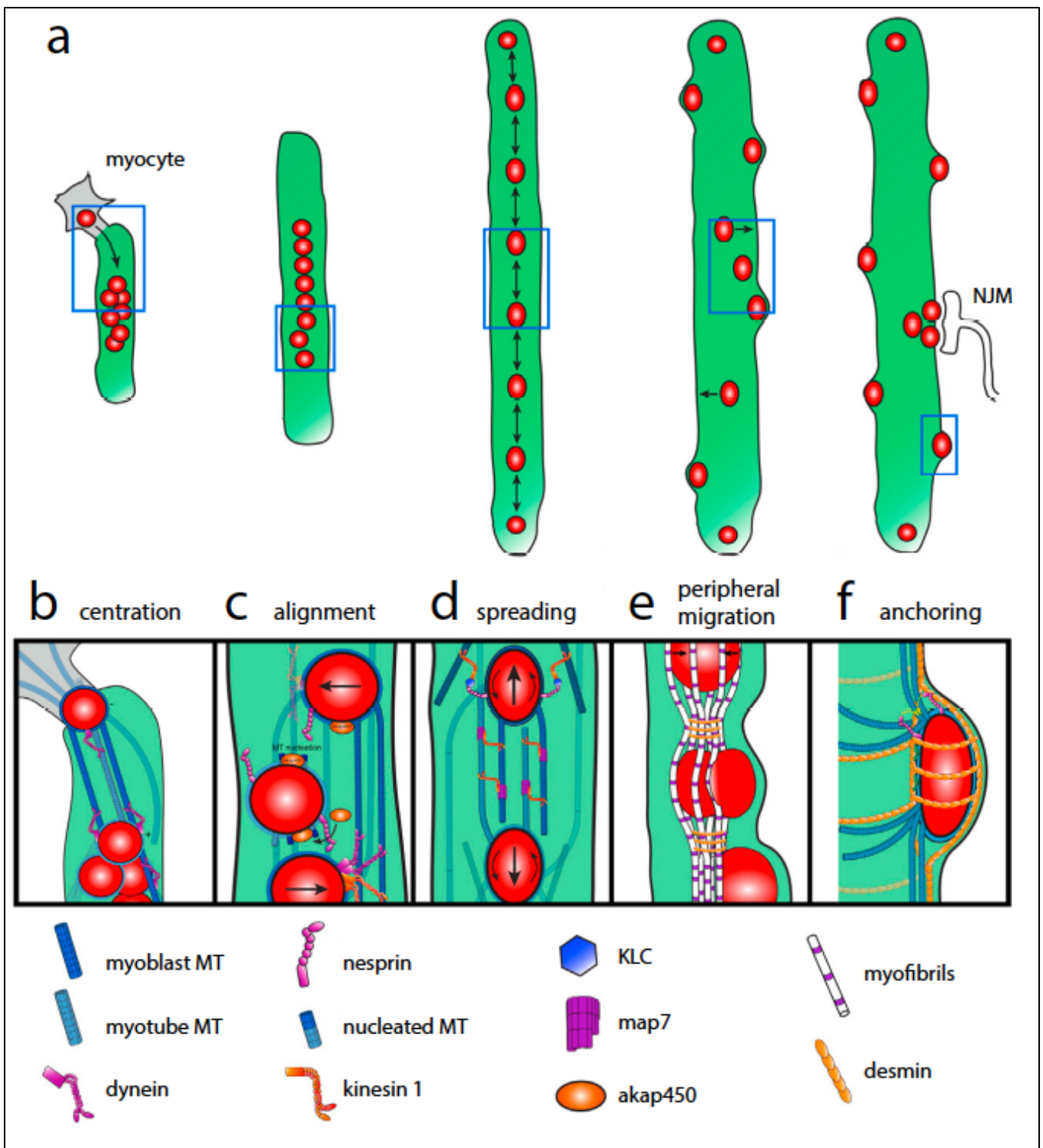


Figure 9. Nuclear positioning during myogenic differentiation.

a) Nuclear movements during myogenesis. Blue rectangles represent zoomed illustrations of B–F.

b) Centration. Nucleus from newly fused myoblast is pulled toward the center of the myotube by dynein and microtubules (MT).

c) Alignment. Nesprin-1 α and PCM-1 recruit centrosomal and motor proteins to the NE.

d) Spreading. Kinesin-1 and Map7 attached to microtubules allow use the anti-parallel microtubular network to spread nuclei along the length of the myotube. Kinesin-1 and kinesin light chain (KLC) at the nuclear envelop walk towards the (+) end of surrounding microtubules to induce nuclear rotation.

e) Peripheral migration. Myofibril crosslinking, contraction and nuclear stiffness variations drive nuclear movement to the periphery. Myofibril crosslinking is mediated by desmin organization at the z-lines.

f) Anchoring. Nesprin organizes an astral microtubular network to anchor nuclei just below the plasma membrane. Anchoring is reinforced by a desmin network. (Roman & Gomes, 2017)

they are cross-linked at the level of the Z line with the help of desmin, which proper organization for cross-linking depends on the Arp2/3 complex and γ -actin. All together, these proteins will act like closing zippers that push nuclei towards the periphery. This process is further enhanced by myofibril contraction and lower nuclear stiffness tuned by local lamin A/C level decrease (Roman et al., 2017). Nuclei can either be aggregated to the myotendinous junction (MJ), involved in stretching, or to the neuromuscular junction (NMJ), involved in contraction, for localized expression of specific proteins. They can also be isolated and evenly distributed at the periphery of myofibers. The two different anchorage mechanisms involved may depend on nucleus fate, but are so far poorly understood (Roman & Gomes, 2017). The mechanisms of nuclear positioning are summarized in **Figure 9**.

1.4.2 The triad: the sarcoplasmic reticulum and the T-tubule system

The triad, a specific structure only found in skeletal muscle, is key for muscle contraction. It is composed of the association of one T-tubule and two terminal cisternae of the sarcoplasmic reticulum (Al-Qusairi & Laporte, 2011).

T-tubules are deep membrane invaginations that insert between myofibrils. Their main role is to induce Ca^{2+} release from the SR, needed for muscle contraction (see chapter 1.3.1). Prior to release, calcium is stored in the SR through a calsequestrin-dependent process (Costello et al., 1986). Calsequestrin is localized at terminal cisternae where its anchorage depends on other proteins such as junctins, triadin and RyR1 (ryanodine receptor 1) (Zhang et al., 1997). It can bind Ca^{2+} with low affinity but in a high capacity manner, allowing high calcium concentration close to release sites (MacLennan & Wong, 1971). Calcium release depends on the tight association between the L-type calcium channel DHPR (dihydropyridine receptor), located at the T-tubule, and RyR1, located at the level of the terminal cisternae of SR (Marty et al., 1994; Rios & Brum, 1987). Action potential spreading into T-tubules triggers DHPR conformational changes, leading to

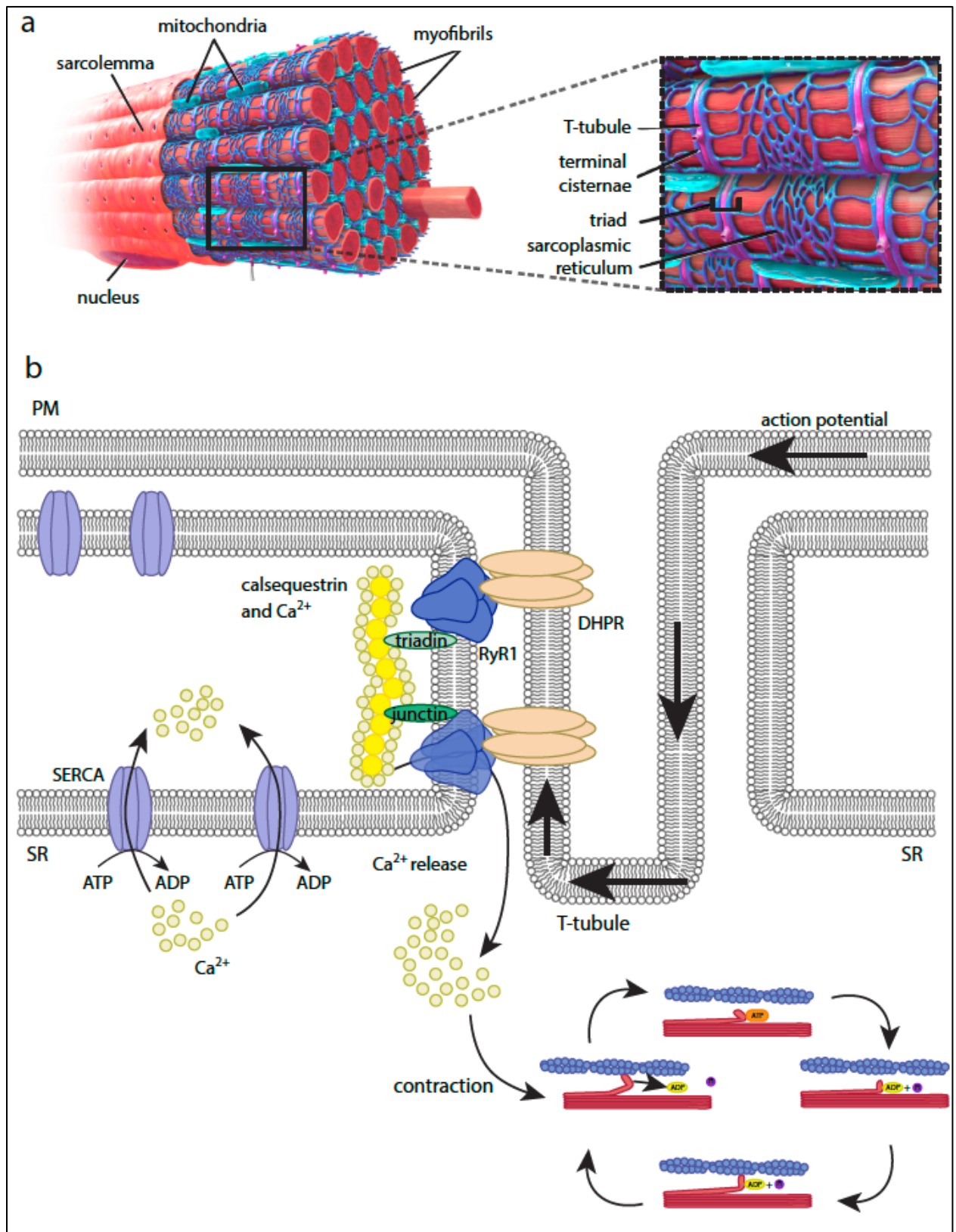


Figure 10. Calcium release and uptake during excitation-contraction coupling controlled by the Triad.

a) Localization of the triad in myofibers, composed of one T-tubule and two terminal cisternae of the sarcoplasmic reticulum (SR) are surrounding. (Blausen.com staff (2014). "Medical gallery of Blausen Medical 2014". WikiJournal of Medicine)

b) At rest, Ca^{2+} is concentrated and associated with calsequestrin, located right under Ryanodine Receptor 1 (RyR1) at SR terminal cisternae through its association with triadins and junctins. Upon excitation, the action potential propagates through the plasma membrane and the T-tubules, where it induces dihydropyridine receptor (DHPR) activation. DHPR is tightly associated with RyR1 and its activation induces RyR1 conformational change, leading to Ca^{2+} release and muscle contraction. After contraction, the excess of Ca^{2+} is re-uptaken in the SR through SERCA (sarco-endoplasmic reticulum calcium ATPase pumps) pumps, in an ATP-dependent process.

RyR1 opening, allowing the massive Ca^{2+} release in the cytoplasm, and subsequent contraction (Marty et al., 1994). Finally, during muscle relaxation, the excess of calcium needs to be re-captured by the SR. This process is mainly handled by sarco-endoplasmic reticulum calcium ATPase pumps (SERCA), located both at terminal cisternae and longitudinal SR (Yu et al., 1993). The mechanism of calcium release and uptake by the Triad is summarized in **Figure 10**.

In addition to T-tubules, the plasma membrane of muscle cells is also highly enriched in a particular membrane invagination called caveolae. The next chapter will describe their constituents, biogenesis and functions. I will then discuss the particular functions of caveolae in the skeletal muscle, as well as their implication in muscle diseases.

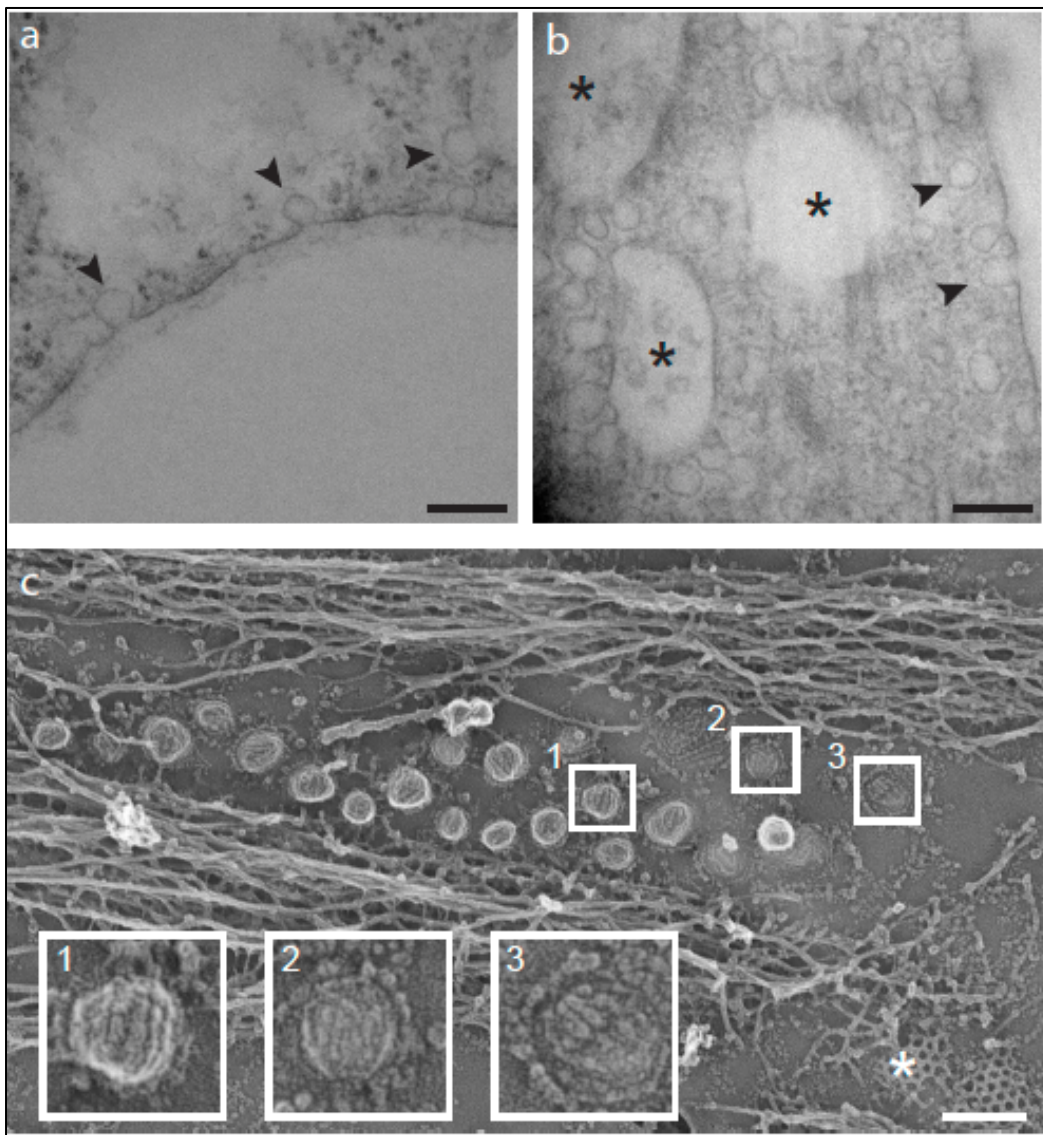


Figure 11. Caveolae observation by electron microscopy.

a, b) Caveolae can adopt different organizations. It can be single caveolae individually connected to the plasma membrane (indicated with arrowheads), or interconnected caveolae forming bigger structures, called rosettes that can still be connected to the plasma membrane (indicated with asterisks).

c) Visualization of the caveolar coat at the plasma membrane of myotubes. Survey view of the cytoplasmic surface of an unroofed mouse myotube presenting caveolae at the plasma membrane. Different types of caveolae structures are apparent, ranging from fully budded (1), circular (2), to flat (3). Scale bar: 500 nm. Scale bar in insets: 50 nm. Adapted from (Lamaze et al., 2017).

2 Caveolae

2.1 Caveolae, specialized plasma membrane invaginations

Caveolae were observed for the first time by electron microscopy in the '50s by analyzing the structure of blood capillaries and the gall bladder epithelium (Palade, 1953; Yamada, 1955). They are defined as 50-100 nm diameter plasma membrane invaginations, and can be distinguished from clathrin coated pits when observed by rapid-freeze deep-etch electron microscopy (Rothberg et al., 1992). In mammals, caveolae are present in all type of cells, with the notable exception of neurons and lymphocytes, although they express caveolin-1, and are particularly enriched in adipocytes, endothelial and muscle cells. Caveolae can be found as single caveolae, associated to specialized structures such as T-tubule in differentiating muscle cells (Parton et al., 1997), or interconnected as a multi-caveolar structure called "rosette" (Pelkmans & Zerial, 2005) (**Figure 11**). After their discovery, it took almost 40 years to identify caveolin-1 (Cav1) as their main protein component (Glenney, 1992; Kurzchalia et al., 1992; Rothberg et al., 1992). This identification was followed by the characterization of two other members of the caveolin family, caveolin-2 (Cav2) and caveolin-3 (Cav3), and was the starting point of a better understanding of caveolae biogenesis and function in cells.

2.2 Caveolae biogenesis

2.2.1 Caveolae protein composition

2.2.1.1 Caveolins

Caveolin-1, the first identified member of the caveolin family, was discovered by two distinct teams at the same time but was thought to be two different proteins, VIP-21

(vesicular integral protein 1) and caveolin-1 (Kurzchalia et al., 1992; Rothberg et al., 1992). It was then established that VIP-21 and caveolin-1 were the same protein, and the name caveolin-1 was kept (Glenney, 1992). Cav1 is a small protein of 21-24 kDa with two isoforms: α -Cav1 and β -Cav1. The β -Cav1 isoform lacks the first 31 amino acids (aa) compared to α -Cav1. Nevertheless, they both have a 33 aa hydrophobic domain, suggested to be a β -sheet inserted in the plasma membrane (Glenney & Soppet, 1992), with the N- and C-terminus facing the cytosol (Dupree et al., 1993; Monier et al., 1995). α -Cav1 (not β -) can be phosphorylated on its Tyrosine 14 (Tyr14) residue upon Rous sarcoma virus, or after insulin or Src activation (Glenney, 1989; Shengwen et al., 1996; Mastick, et al., 1995). The consequences of this modification will be further discussed in chapter 2.2.3. Cav1 can also be phosphorylated on its serine 80 (ser80) residue, which allows Cav1 binding to ER (endoplasmic reticulum) proteins and its secretion in the particular case of regulated secretion by pancreatic cells (Schlegel et al., 2001). Controlled Ser80 phosphorylation is also crucial for proper caveolae formation and shape (Ariotti et al., 2015). Cav1 presents also three palmitoylation sites (Cys133, Cys153 and Cys156) which are not necessary for membrane anchorage but may influence caveolin oligomerization (Dietzen et al., 1995; Monier et al., 1996). Using velocity sucrose gradient centrifugation, Cav1 was shown to form 300 kDa high molecular complexes. Caveolin proteins can form homo- or hetero- oligomers of α -/ β -Cav1 and Cav2 (Root et al., 2015; Sargiacomo et al., 1995). This phenomenon occurs through the Cav1 oligomerization domain (residues 61-101). The oligomerization process and its purpose will be further detailed in chapter 2.2.3.4.

Few years after Cav1 discovery, a potential homologue of Cav1 was found by nucleotide alignment. It allowed the identification of the muscle specific isoform that was first called M-caveolin, to be later renamed caveolin-3 (Cav3) (Way & Parton, 1995). Compared to α -Cav1, Cav3 has 65% of identity and 85% of similarity, and lacks the first 27 aa. Its expression is restricted to cardiac, smooth and skeletal muscle, and is only expressed upon differentiation in skeletal muscle. Because it lacks the Tyr14 present in Cav1, it is

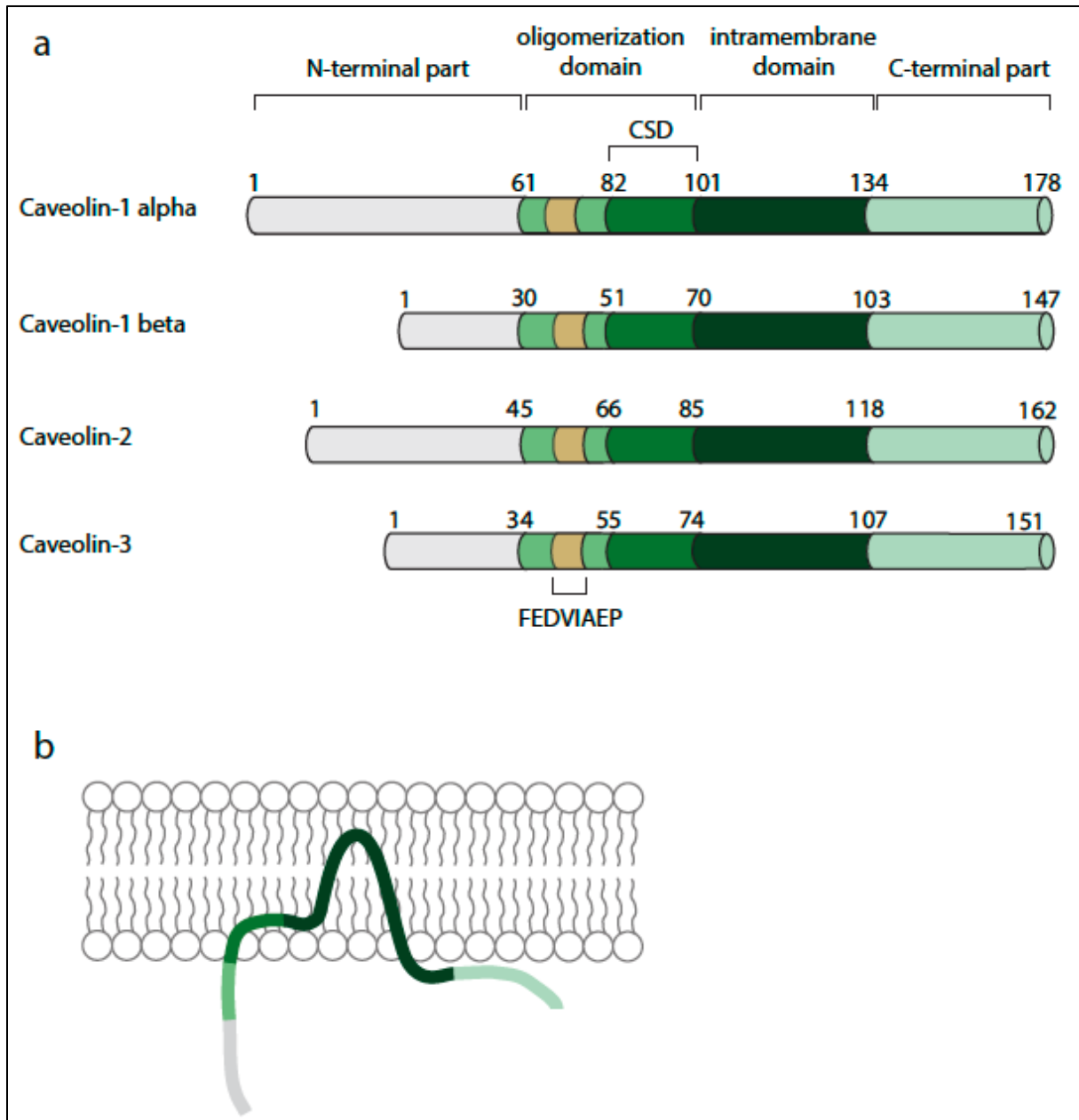


Figure 12. Caveolin protein family domains and insertion in the plasma membrane.

a) Caveolin protein family exhibit several very conserved domains: the oligomerization domain containing the 8aa stretch signature sequence (FEDVIAEP) and the caveolin scaffolding domain (CSD), and the intramembrane domain. Size variability within the caveolin family is due to length difference of the N-terminal part of caveolins.

b) Caveolin 1 (Cav1) is inserted in the plasma membrane through its intramembrane domain, with the N- and C-terminal parts facing the cytosol, conferring a hairpin shape. In addition, three palmitoylation sites allow anchoring and stability of the protein at the plasma membrane. Based on (Parton & del Pozo, 2013).

not phosphorylated, but it still shares a lot of common features with Cav1. Indeed, Cav3 is palmitoylated, has the same conserved hydrophobic domain and is able to oligomerize (Tang et al., 1996; Way & Parton, 1995). Furthermore, in cardiac myocytes, one of the few cells expressing both Cav1 and Cav3, it has been shown that these caveolins could form hetero-oligomers (Volonte et al., 2008).

The last member of the family, and the least studied, is caveolin-2. Based on the purification of membrane fractions enriched in caveolae, it was first defined as a protein with 38% identity and 58% similarity with Cav1. Furthermore, a specific stretch of 8 aa (FEDVIAEP) in the N-terminal part, extremely conserved with Cav1, and found later to be also present in Cav3, was found (Scherer et al., 1996; Tang et al., 1996). It was thus suggested to be the caveolin family signature. Cav2 exists under two isoforms, α -Cav2 and β -Cav2, but not much is known about their potential differences in expression or function. Compared to α -Cav1, α -Cav2 and β -Cav2 lack the first 16 aa and 29 aa respectively. Cav2 localizes at caveolae structures, and colocalize with Cav1. As it is expressed in skeletal muscle cells, contrary to Cav1, it also colocalizes with Cav3. It has the capability to dimerize but not oligomerize further, and can form hetero-oligomers with other caveolin isoforms (Scherer et al., 1996, 1997). As Cav1 and Cav3, α -Cav2 is also triple palmitoylated and although α -Cav2 lacks the Tyr14, it can still be phosphorylated on its Tyr19 and Tyr27 residues upon insulin stimulation (Kwon et al., 2015). Interestingly, there is no evidence that these residues can also be phosphorylated in the other caveolins.

In summary, all three members of the caveolin family have a very similar structure, composed of an N-terminal part containing a very conserved stretch of 8 aa, a very conserved 33 aa hydrophobic region (residues 102-134 of Cav1) with N- and C-terminus facing the cytosol. This allows a particular "hairpin" conformation of the protein, inserted in the plasma membrane (**Figure 12**).

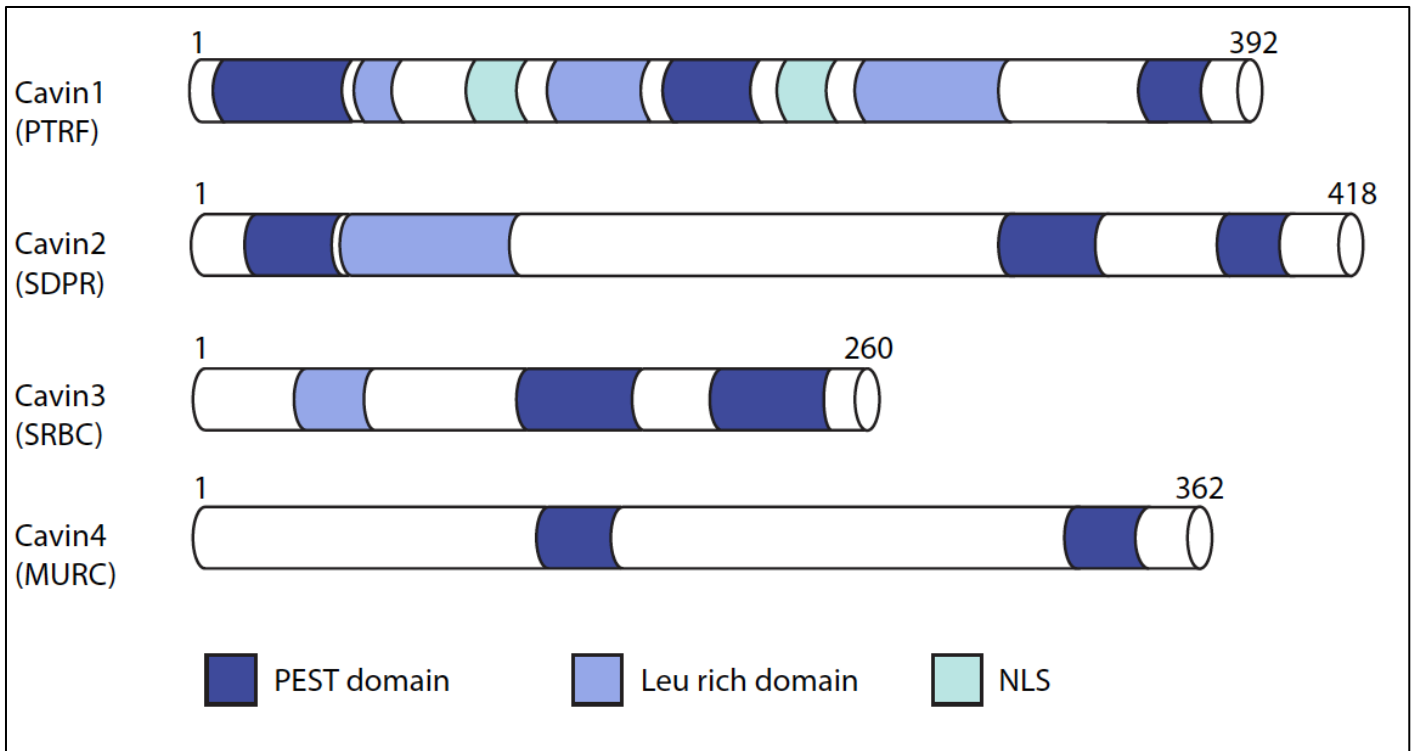


Figure 13. Cavin structure.

Cavins are heterogeneous in size but they all contain PEST and Leucine rich domains for potential interaction with other proteins. Cavin-1 is the only member of the cavin family with a NLS sequence, favoring nuclear translocation. Based on (Bastiani et al., 2009).

2.2.1.2 Cavins

In mammals, there are four members in the cavin family, cavin-1, 2, 3 and 4. Cavin-1 was the first to be identified when the Trandum-Jensen group screened for antibodies recognizing proteins localized in caveolae structures (Vinten et al., 2001). A 60 kDa protein was found to be highly enriched at caveolae, but was not identified. Three years later, PTRF (Polymerase I and transcript release factor) was found to be a major protein of caveolae by mass spectrometry on caveolae enriched membrane fractions from human primary adipocytes (Aboulaich et al., 2004). Shortly after, the Trandum-Jensen group confirmed that the 60 kDa protein they identified before corresponds to PTRF and showed its localization to caveolar structures. PTRF was then renamed into cavin-1 (Vinten et al., 2005).

Cavin-2 and cavin-3 were originally discovered as two proteins involved in serum deprivation, SDPR (serum deprivation-response protein) and SRBC (sdr-related gene product that binds to c-kinase) (Gustincich & Schneider, 1993; Izumi et al., 1997). SDPR was shown quite early to be associated to caveolae, together with PKC α (protein kinase C α) (Mineo et al., 1998), and SDPR and SRBC were also found in the same mass spectrometry analysis that identified cavin-1 (Aboulaich et al., 2004).

All three proteins share common features as they contain leucine zipper domains and PEST (sequences enriched in proline, glutamic acid, serine and threonine) domains (**Figure 13**). They were thus considered to belong to the same protein family. SDPR and SRBC were then renamed cavin-2 and cavin-3 respectively. All cavins contain the HR1 and HR2 domain (Helical region 1 and 2), allowing oligomerization (Kovtun et al., 2014). Cavins, through interaction with the HR1 domain, can form trimers consisting of either three cavin-1 or two cavin-1 associated with one cavin-2 or one cavin-3 protein (Kovtun et al., 2015; Lamaze et al., 2017). The recruitment of cavin complexes to caveolae will be further described in chapter 2.2.3.3.

MURC (muscle-restricted coiled-coil protein)/cavin-4 was later identified by sequence homology screening with 29% identity and 49% similarity to cavin-1, 24% identity and 42% similarity to Cavin-2, and 20% identity and 39% similarity to cavin-3 in mice (Bastiani et al., 2009). Like Cav3, Cavin-4 expression is restricted to cardiac and skeletal muscle, and highly increased upon muscle cell differentiation. Therefore, cavin-4 is considered as the muscle specific isoform of the cavin family, even though the other cavins are still present.

Caveolins and cavins are the main protein components of the caveolar coat, and are both required at the plasma membrane for the biogenesis of *bona fide* caveola structures (see chapter 2.2). Nevertheless, a few more accessory proteins are necessary for the stability and dynamics of caveolae.

2.2.1.3 Accessory proteins

Although they are not essential for the formation of caveolae, the dynamin-2 GTPase, the F-BAR protein pacsin2 (protein kinase C and casein kinase substrate in neurons protein 2, also called syndapin II) and the EHD2 ATPase (EH-domain containing 2) proteins could be found at the neck of caveolae (Hansen et al., 2011; Henley et al., 1998; Morén et al., 2012; Stoeber et al., 2012). While dynamin-2 is involved in the endocytosis of caveolae (further detailed in chapter 2.2.1), pacsin2 and pacsin3 influence caveolae morphology, and EHD2 the dynamics of caveolae (both further detailed in chapter 2.2.4.3).

2.2.2 Caveolae, a specific lipid composition

Caveolae have been initially defined as a specific type of lipid rafts, which are nanodomains of the plasma membrane, enriched in sterols, sphingolipids and ordered assembly of specific proteins (Simons & Sampaio, 2011; Simons & Ikonen, 1997). Similarly

to the classical lipid rafts, they are enriched in cholesterol and sphingolipids, which confers them detergent treatment resistance, a property defining lipid rafts (Simons & Toomre, 2000). But it is now well defined that caveolae are not lipid raft, as their lipid composition is not limited to cholesterol and sphingolipids, and that these lipids are not as dynamic as they would be in a classical lipid raft. Indeed, caveolae also contain other lipids such as sphingomyelin, glycerophospholipids, phosphatidylserine (PS), phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂, or PIP₂) and glycosphingolipids such as GD3 and at a lesser extent, GM3, GM1 and GD1a (Iwabuchi et al., 1998; Örtegren et al., 2004; Sonnino & Prinetti, 2009). The lipid composition of caveolae is partly due to the capacity of Cav1 to bind cholesterol probably through its CRAC (cholesterol recognition/interaction amino acid consensus) motif with a ratio of 1:1, but also sphingolipids such as GM1 (Epanand et al., 2005; Fra et al., 1995; Murata et al., 1995).

2.2.3 Caveolae formation, a step by step process

2.2.3.1 Minimum requirements

Although a lot of progress has been made to determine caveolae biogenesis, this process is still poorly understood. To be achieved, the formation of proper caveolae at the plasma membrane needs essential elements. It was observed quite early that cholesterol depletion leads to caveolae flattening, suggesting a predominant role of this lipid in its biogenesis process (Rothberg et al., 1992; Rothberg et al., 1990). Lipids are not the only important feature of caveolae formation. For long, Cav1 (and Cav3 in muscle cells) were thought to be the only necessary proteins. Indeed, in lymphocytes, one of the few cells devoid of Cav1, no caveolae structures can be detected (Fra et al., 1994). Lack of caveolae was also observed in all tissues in CAV1 KO mice, at the exception of skeletal muscle tissue (Drab, 2001; Razani et al., 2001). And in CAV3 KO mice, no caveolae structure were observed in cardiac and muscle cells (Galbiati et al., 2001). Supporting these observations, the heterologous expression of Cav1 was sufficient to induce *de*

novo caveolae formation in lymphocytes (Fra et al., 1995) and more surprisingly in bacteria (Walser et al., 2012). Ten years ago, the identification of cavin-1 changed the established dogma. Indeed, CAVIN-1 KO mice show a global loss of caveolae, even in skeletal muscle, as it is ubiquitously expressed (Liu et al., 2008). Because cavin-1 and Cav1 are usually transcriptionally co-regulated, it can be hard to distinguish the impact of cavin-1 loss compared to Cav1 loss. To overcome this issue, we can take advantage of PC3 cells and cells from the notochord of zebrafish have the particularity of expressing Cav1 but not cavin-1. No caveolae structure can be observed in these cells, demonstrating unambiguously that cavin-1 is indeed necessary for caveolae formation (Hill et al., 2008).

2.2.3.2 Caveolin oligomerization and trafficking to the plasma membrane

Caveolae formation also requires sequential processes, starting with a first round of Cav1 oligomerization in the ER. Then, Cav1 oligomers are rapidly (within 15 min) exported to the Golgi complex in a COPII dependent manner, and this step requires the $_{67}DXE_{69}$ motif of Cav1 (Hayer et al., 2010; Monier et al., 1995). In this organelle, a cholesterol dependent oligomerization induces conformational changes and a loss of Cav1 apparent mobility, probably due to oligomers association with lipids. At last, caveolin-enriched vesicles, containing 140-160 Cav1 molecules, are exported to the plasma membrane (Hayer et al., 2010; Pelkmans & Zerial, 2005). Once at the plasma membrane, although Cav1 palmitoylation is not required for the formation of the oligomers, it seems to be important for their stabilization (Monier et al., 1996). Cav1 oligomers will then cluster several lipid species such as PIP2 and PS (Sonnino & Prinetti, 2009), which will further recruit others caveolae components, mainly the cavin coat.

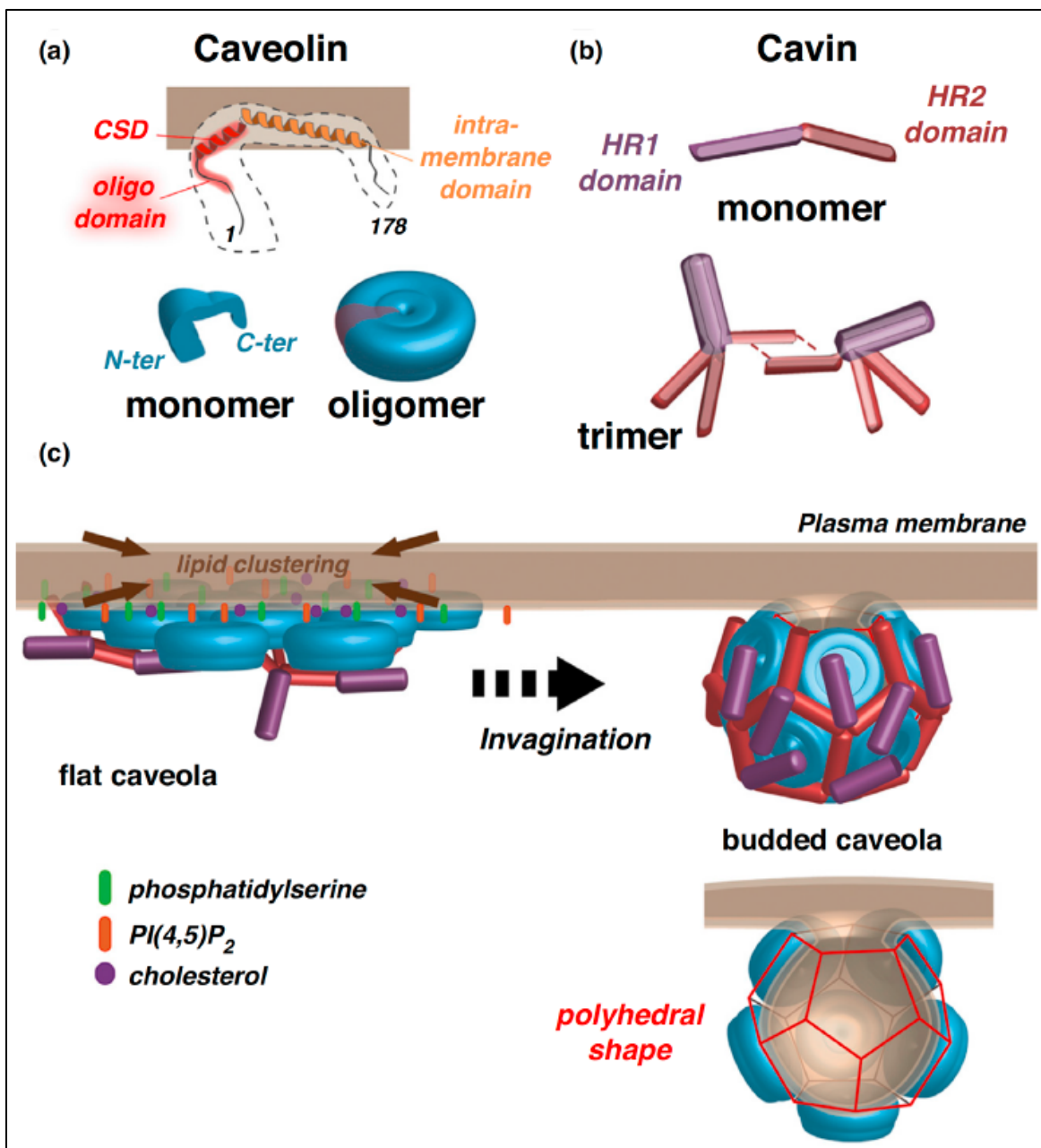


Figure 14. Putative model for caveolar coat assembly and organization.

a) Schematic model of Cav1 topology. Cav1 is inserted into the plasma membrane through the caveolin scaffolding domain (CSD; red), an amphipathic helix part of the oligomerization domain (diffuse red), and through a second amphipathic helix, the intra-membrane domain (orange). Based on Cav3 ternary structure, Cav1 monomers may assemble as a disk-shaped oligomer with the C-terminal part oriented toward the center.

b) Cavin monomers exhibit two helical rich domains, HR1 and HR2, that may form coil-coiled structures. Cavins, through interaction with the HR1 domain, can form trimers consisting of either three cavin-1 or two cavin-1 associated with one cavin-2 or one cavin-3 protein. The cavin-1 isoform could be responsible for a more complex assembly through the coiled-coil domain 2 (cc2) sequence in the HR2 domain.

c) At the plasma membrane, Cav1 oligomers cluster specific lipids such as cholesterol, PI(4,5)P₂ and phosphatidyl serine involved in the recruitment of cavin trimers. This is followed by caveola invagination, a process not completely understood. It has been recently suggested that the overall architecture of the caveolar coat made of caveolins and cavins would best fit with a polyhedron structure. In this model, Cav1 oligomers position on each pentagonal face and cavin complexes align with the vertices and cover the Cav1 oligomers. (Lamaze et al., 2017)

2.2.3.3 Cavins recruitment and caveolae invagination

Cavin-1 is a cytosolic protein that has been shown to be recruited on caveolin not at the level of the Golgi complex but only at the plasma membrane, once Cav1 oligomers are present (Hill et al., 2008). First, cavin recruitment has been suggested to occur via caveolins (Bastiani et al., 2009), but recent studies suggests that they are rather recruited via PS that are actually clustered in caveolae (Kovtun et al., 2014; Stoeber et al., 2016). This is supported by a recent study reporting a crucial role for PS in Cav1-cavin-1 association and caveolae formation (Hirama et al., 2017). Cavin-1 plays a central role, as it is necessary for the invagination of caveolae (Hill et al., 2008) and the recruitment of both cavin-2, -3 and -4 (Bastiani et al., 2009). Cavin-2 seems to play a role in the shaping of caveolae, as its overexpression leads to the formation of enlarged caveolae. Cavin-3 is suggested to have a role in caveolae budding to form caveolar vesicles (Nabi, 2009). Finally, not so much is known about Cavin-4 involvement in caveolae formation (Lamaze et al., 2017).

The putative model of caveolar coat assembly and organization is summarized in **Figure 14**.

2.2.3.4 Recruitment of accessory proteins

Although not essential for the formation of caveolae, one last set of proteins can be recruited to caveolae and influences their morphology and dynamics. The GTPase dynamin2 was shown to be located at the neck of caveolae, allowing their budding and fission (Pelkmans et al., 2002). This will be further detailed in chapter 2.2.1.

Two other proteins, members of the same protein family, pacsin2 (syndapin II) in non-muscle tissues and pacsin3 (syndapin III) in muscle tissue, have been showed to be associated caveola structure. While pacsin2 recruitment mechanism to the neck of

caveolae is still unknown, its depletion or dissociation from caveolae is followed by caveolae number decrease and increased caveolin and cavin complexes at the plasma membrane (Hansen et al., 2011; Senju & Suetsugu, 2015). The same results were obtained upon depletion of pacsin3 in muscle tissue (Seemann et al., 2017). This suggests that pacsin2 and 3 are involved in the morphology of caveolae. Furthermore, together with EHD2 (EH-domain containing 2), another caveolae associated protein, pacsin2 protects caveolae from endocytosis by inhibiting the recruitment of dynamin2 (Senju et al., 2015).

EHD2 recruitment to the neck of caveolae requires its oligomerization and ATP binding. Although EHD2 is not required for the formation of caveolae, it allows the stability of static caveolae at the plasma membrane, as its depletion leads to a higher fraction of mobile caveolae diffusing in the membrane (Morén et al., 2012; Stoeber et al., 2016). Also, EHD proteins allows the formation of multi-caveolar structures (Yeow et al., 2017).

2.3 Caveolae functions

Since their discovery, many efforts have been put to understand the functions of caveolae in cells. Because caveolae are present in almost every type of cells, and are highly enriched at the plasma membrane of specialized cells, it has always been thought to have an important role. Thus, it is not surprising that many functions, further detailed in this chapter, have been associated with caveolae.

2.3.1 Caveolae and cell trafficking

Due to their morphological resemblance to clathrin coated pits (CCP) and their membrane localization, caveolae were first suspected to play a role in internalization. They were first described to be involved in transcytosis in muscle capillaries (Simionescu et al., 1975). This mechanism was proposed to be important for the transport of fluid-

phase or receptor-mediated albumin and LDL (low density lipoprotein) uptake in endothelial cells (Frank et al., 2009; Ghitescu et al., 1986; Schubert et al., 2001). While this specialized mechanism seems to be restricted to endothelial cells, caveolae mediated endocytosis appears to be quite ubiquitous as a caveolae fraction has been observed as not being static at the plasma membrane. Some exogenous cargos can trigger these events like SV40 virus or Cholera Toxin (CTx) (Anderson et al., 1996; Orlandi & Fishman, 1998; Tran et al., 1987). Some caveolae can undergo cycles of appearance and disappearance at the PM, without losing their caveolar coat, in a “kiss-and-run” manner (Pelkmans & Zerial, 2005). As described previously, the dynamin2 GTPase can be recruited, although at a low extent, to the neck of caveolae, together with actin, and provokes fission from the plasma membrane after GTP hydrolysis (Oh et al., 1998; Pelkmans et al., 2002). Endocytosed caveolae then fuse with early endosomes, as it has been recently confirmed that caveolae structures fusing with early endosomes were indeed caveolae which had budded from the plasma membrane (Shvets et al., 2015). Other internal structures positive for Cav1 were thought to be an alternative pathway, independent from endosomes, and were called caveosomes. But it turned out that caveosomes actually correspond to late endosomal and multivesicular bodies structures where Cav1 when overexpressed accumulates, gets ubiquitinated and degraded (Hayer et al., 2010). Caveolae and more specifically caveolae components are also tightly linked to the CLIC/GEEC pathway (clathrin-independent carriers/GPI-AP enriched early endosomal compartment). Indeed, it was shown that Cav1, Cav3, cavin1 and cavin3 could inhibit the CLIC/GEEC pathway, independently from caveolae structures. Furthermore, Cav1 and Cav3 seem to act through the caveolin scaffolding domain (CSD) since CSD mimicking peptides are sufficient to induce the same phenotype (Chaudhary et al., 2014). Apart from virus and toxins, some lipids can also trigger endocytosis of caveolae structures such as cholesterol and glycosphingolipids (GSL), notably lactosylceramide (Pagano, 2003; Sharma et al., 2003). In adipocytes, upon cholesterol addition and Src kinase activation, caveolae can also be endocytosed and

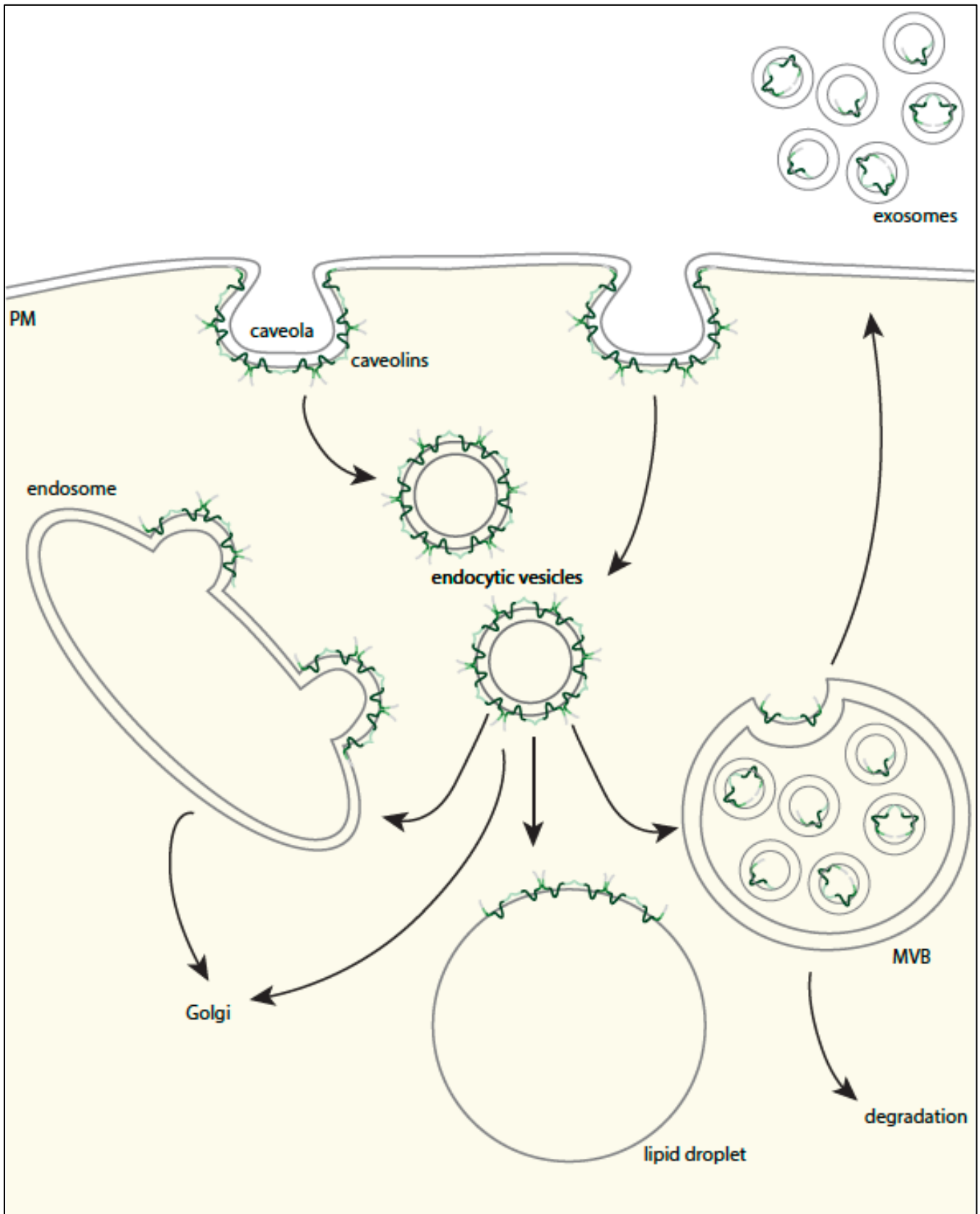


Figure 15. Fate of Cav1 following caveolar endocytosis.

Depending on the cargo, caveolae and Cav1 can be endocytosed to further fuse with endosomes, the Golgi complex, lipid droplets or multivesicular bodies (MVBs). Cav1 targeting to endosomes or to the Golgi complex is mostly associated with its recycling. Cav1 targeting to lipid droplets in adipocytes is involved in lipid storage. Finally Cav1 targeting to MVBs is either associated with degradation, or with exosome association for potential further cell-cell communication.

targeted to lipid droplets, allowing the maintenance of free cholesterol in these structures (Le Lay et al., 2006). Until now, no specific caveolae cargo has been identified. As mentioned before, in some cases, following caveolae endocytosis, Cav1 accumulates to multivesicular bodies (MVBs) and is degraded. But we could also hypothesize that it associates with extracellular vesicles (EVs) and gets secreted. This idea is supported by the fact that Cav1 has been found in EVs derived from melanoma cells, but also in prostasomes, which are prostate cells specific EVs that derive from intracellular structures similar to MVBs (Llorente et al., 2004; Logozzi et al., 2009). More recently, it was shown that Cav1 was expressed in adipocyte-derived EVs. Two types of EVs are secreted, small and large EVs (sEVs and lEVs), and Cav1 exclusively associates with lEVs that are enriched in PS (Durcin et al., 2017). Once secreted, EVs can be uptaken by other cells, or interact with cells by presenting protein at their surface, as it is the case for the MHC (major histocompatibility complex) class II and ICAM-1 (intercellular adhesion molecule 1) in dendritic cell derived EVs (Segura et al., 2005). But the fate of Cav1 containing EVs is not yet known.

The different fates of endocytosed caveolae are summarized in **Figure 15**.

2.3.2 Caveolae and lipid homeostasis

Due to its particular lipid composition, it has been proposed that caveolae could regulate cellular lipid homeostasis by different means. And indeed, caveolae can regulate fatty acid transport through plasma membrane (Meshulam et al., 2006) and sphingolipid internalization from this compartment (Shvets et al., 2015). Caveolae were even proposed to host triacylglycerol (TG) synthesis (Öst et al., 2005). Inside the cell, caveolae can also influence lipid sorting, as Cav1 and Cav2 can be associated with lipid droplets, an organelle specialized in lipid storage (Fujimoto et al., 2001; Ostermeyer et al., 2001; Pol et al., 2001). In adipocytes, Cav1 associates with lipid droplets during their maturation in a Src kinase dependent manner, and can be triggered by cholesterol

addition, as mentioned in the previous chapter, or when it accumulates in the ER (Blouin et al., 2008; Le Lay et al., 2006; Ostermeyer et al., 2001). In addition to the regulation of free cholesterol levels, Cav1 has also shown to be crucial for lipid droplet composition. Indeed, Cav1 deficient lipid droplets are smaller and have decreased amount of surface PS and lysophospholipids suggesting a role for lipid sorting and lipid droplet size regulation (Blouin et al., 2010). Finally, through their ability to regulate signaling pathway (detailed in the next chapter), caveolae play also a role in lipolysis through modulation of adrenergic receptors (Cohen et al., 2004) or in lipid storage through the insulin pathway (Kim et al., 2008; Mastick et al., 1995; Yamamoto et al., 1998).

2.3.3 Caveolae and signaling

Based on the early observations that caveolin is a substrate of Src kinase, and that G-coupled proteins are located in caveolae, it has been proposed that caveolae could be involved in signaling events (Lisanti et al., 1994). Since then, the number of papers linking caveolae and/or caveolar components to signaling pathways has significantly grown. Indeed, a large panel of signaling molecules have been shown to be located in caveolae, among them eNOS (endothelial nitric oxide synthase), H-ras, EGFR (epidermal growth factor receptor), TGF- β (transforming growth factor β) or insulin (reviewed in Lamaze et al., 2017, see annex). How caveolae can regulate signaling events is a question that is still debated. It seems that caveolae could actually regulate signaling by different means (reviewed in Lamaze et al., 2017).

The first one relies on the direct interaction of caveolin with signaling molecules, inducing most of the time a negative regulation. Indeed, the Lisanti group identified a specific region (residues 61-101) of caveolin that was necessary and sufficient for the interaction with heteromeric G proteins, Src tyrosine kinase and H-ras (Li et al., 1995; Li et al., 1996). But later, the dissection of caveolin / eNOS interaction using truncated form of Cav1 or Cav3 allowed to minimize the region to residues 82-101 for Cav1 or 55-74 for

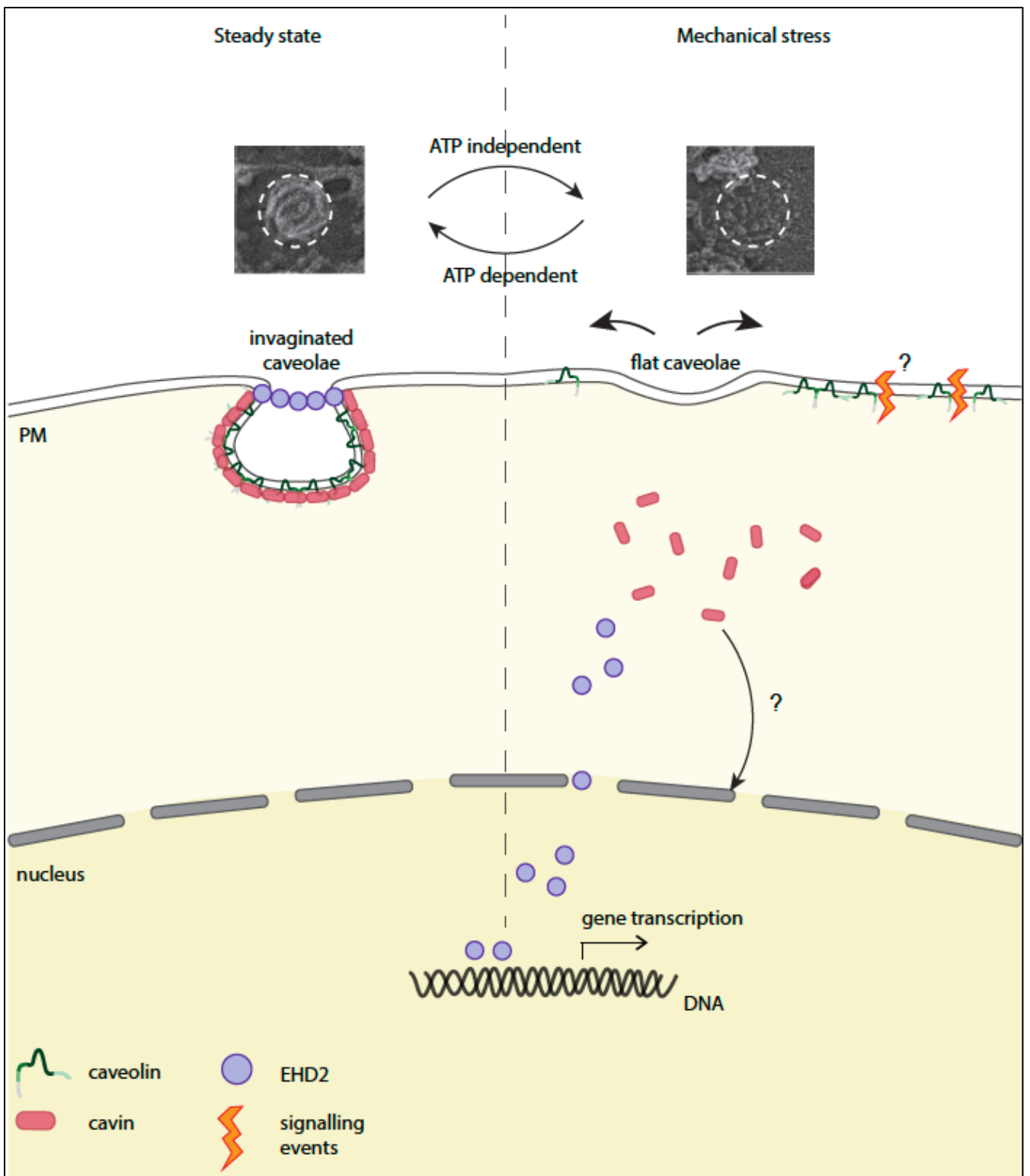


Figure 16. Caveolae components behavior after caveolae flattening.

In resting condition (steady state), caveolae are invaginated at the plasma membrane. Upon mechanical stress, caveolae flatten out, releasing their compounds either in the cytoplasm for cavins and EHD2, or in the plasma membrane for caveolins. Cavin-1 may go to the nucleus and regulate the expression of some genes, as it is the case for EHD2. It could also interact with potential partners in the cytoplasm through its PEST domains, and regulating their activity. Cavin-2 and -3 probably remain in the cytoplasm but nothing is known about their fate after caveolae flattening. Caveolins could interact with potential partners, possibly through a CSD-CBM interaction and regulate some signaling pathways. Based on (Nassoy & Lamaze, 2012). Deep-etched EM images from (Sinha et al., 2011).

Cav3. This particular region is called the caveolin scaffolding domain (CSD) (Garcia-Cardena et al., 1997). Using mimetic cell penetrating peptides, it was shown that the CSD was sufficient to inhibit eNOS activity in cells. Directed point mutagenesis revealed that F92 Cav1 residue has a major role in the inhibition process (Bernatchez et al., 2005). Using phage display, three potential caveolin binding motif (CBM) were identified, all based on an aromatic rich sequence: $\phi X\phi XXXX\phi XX$, $\phi XXXX\phi XX\phi$ and $\phi X\phi XXXX\phi XX\phi$ (ϕ for Trp, Phe, or Tyr and X for any amino acid) (Couet et al., 1997; Garcia-Cardena et al., 1997). Interestingly, a large amount of proteins, already described to be associated with caveolae have a putative CBM. But this hypothesis of interaction between CSD and CBM has been questioned because of the poor stringency of CBM sequence and its potential molecular inaccessibility to the CSD in several cases (Byrne et al., 2012; Collins et al., 2012). New insights into Cav1 insertion in the membrane suggest that the CSD could still be accessible but the question remains open (Liu et al., 2016) (**Figure 16**). In this context, mechanisms facilitating CSD accessibility have been proposed, such as Cav1 Tyr14 phosphorylation (Shajahan et al., 2012). Another interesting hypothesis is the potential role for “free” caveolins (i.e. not associated to caveolae). Up to now, there is no clear evidence that Cav1 can be free at the plasma membrane in resting conditions, but the release of free caveolins can be achieved through flattening of caveolae upon mechanical stress (Nassoy & Lamaze, 2012; Sinha et al., 2011) (**Figure 16**). This hypothesis could make the link between signaling and caveolae mechanics that was suggested for some pathways, such as the Src kinase, RhoA, EGFR/Akt and ERK pathways (Gervásio et al., 2011; Kawabe et al., 2004; Kawamura et al., 2003; Sedding et al., 2005).

Apart from the direct interaction between caveolin and its potential partner, the caveolae itself can play a role in the regulation of signaling events. Indeed, because of its particular lipid composition, it has been suggested to cluster signaling molecules. For instance, calcium pumps and the insulin receptor need to be located in caveolae to function properly (Fecchi et al., 2006; Fujimoto, 1991; Guo et al., 2015). Also,

reorganization of plasma membrane lipids upon Cav1 depletion leads to defects in H-Ras clustering and signaling (Ariotti et al., 2014). Caveolae could also potentially regulate signaling pathways through its endocytic properties, in a fashion similar to what has been described for the clathrin mediated endocytosis pathway (Gonnord et al., 2012).

2.3.4 Caveolae, membrane tension buffering and mechanoprotection

Caveolae are particularly enriched in cells constantly exposed to mechanical stress such as adipocytes, endothelial and muscle cells. While endothelial cells are subjected to shear stress applied by the blood flow, adipocytes undergo swelling and muscle cells repeated cycles of elongation and relaxation. It is thus not surprising that caveolae have been associated early with cell mechanics. The first evidence came with the observation of “opening” of the caveola neck upon skeletal muscle elongation in frogs (Dulhunty & Franzini-Armstrong, 1975). A similar phenotype was observed in smooth muscle cells freeze fracture preparations of the sea slug *Aplysia californica* that were stretched *in vitro* (Prescott & Brightman, 1976). Extensive tool developments to study caveolae and collaboration with physicists allowed our laboratory to definitely establish a new role for caveolae as major mechanosensors that control the immediate response to membrane tension increase in cells. This process, ATP and actin independent, consist in the rapid flattening of the caveolar structure, which results in the release of its components either in the plasma membrane (caveolins) or in the cytosol (cavins). This allows the buffering of increased membrane tension induced by mechanical stress (Sinha et al., 2011). Upon caveolae flattening, GSL packing is lost but very little is known on the other caveolae lipids fate (Gervásio et al., 2011). More recent data obtained in the laboratory show that the accessory protein EHD2 could be translocated to the nucleus after a mechanical stress, where it would act as a transcription factor (Torrino et al., submitted). Caveolae disassembly is reversible and caveolae reassembly requires both ATP and actin (Sinha et al., 2011) (**Figure 16**).

As caveolae disassembly allows buffering of membrane tension increase, it could play a role in cell mechanoprotection, which was confirmed by a higher membrane rupture amount in human muscle cells bearing a CAV3 mutation associated with muscle disease. Interestingly, in the last few years, several studies confirmed the initial findings by Sinha (2011) and showed that caveolae were crucial for mechanoprotection *in vitro* and *in vivo*. Indeed, a lack of caveolae lead to increased susceptibility to acute damage in endothelial cells, cell damage and impaired function of skeletal muscle in zebrafish, and impaired notochord integrity in developing zebrafish (Cheng et al., 2015; Garcia et al., 2017; Lim et al., 2017; Lo et al., 2015).

In addition to cell mechanoprotection, caveolae was also suggested to play a role in cell repair after cell damage. Caveolae could reseal the plasma membrane when damaged through accumulation process at the damage site and endocytosis (Corrotte et al., 2013). Caveolae linked membrane repair has been exclusively studied in muscle tissue, and will be further detailed in chapter 3.1.

2.4 Caveolae related diseases

As mentioned, caveolae have a pleiotropic role in cells. It is thus not surprising that the loss and deregulation of caveolae or its components have been associated with many different diseases. Because it is very difficult to distinguish between the functions of caveolae versus the caveolar components, most of the studies related to caveolae and diseases are based on caveolar component deficiencies or mutations (reviewed in Lamaze et al., 2017, see annex ; Le Lay & Kurzchalia, 2005). I chose to focus this chapter on caveolae related diseases in adipose and vascular tissues, normally highly enriched in these structures, but also in cancer. I will then dedicate the last chapter to caveolae's involvement in muscle related diseases, the main focus of my PhD work.

2.4.1 Lipodystrophy

Both Cav1 and cavin-1 deficient mice exhibit a lipodystrophic phenotype. Cav1 deficient mice are usually smaller and lean, with adipose atrophy observed with aging (Cohen et al., 2003; Razani et al., 2002). This reduced adipose tissue should result from decrease triglyceride and fatty acid uptakes, as these lipids are found in higher concentrations in blood together with decreased level of circulating leptin. Thus, these animals present resistance to diet-induced obesity (Razani et al., 2002). Furthermore, Cav1 deficient mice are insulin resistant, consistent with Cav1 acting as an activator of the insulin receptor (Yamamoto et al., 1998). Indeed, Cav1 deficiency leads to the degradation of the insulin receptor beta unit, which can be rescued by simple addition of a CSD mimicking peptide. Interestingly, Cav2 null mice do not have such a phenotype (Cohen et al., 2003). As the major effect of insulin uptake in adipocytes is to accumulate lipids and avoid lipolysis (Londos et al., 1999), its impairment in Cav1 deficient mice should also participate to their lipodystrophic phenotype. Cavin-1 deficient mice exhibit the same phenotypes as Cav1 deficient mice. The only difference resides in the earlier appearance of adipocyte abnormalities in cavin-1 rather than in Cav1 deficient mice (Liu et al., 2008).

Interestingly, these findings were followed by the identification of a homozygous non sense CAV1 mutations (p.Glu38X, stop codon) in a patient suffering from Berardinelli-Seip congenital lipodystrophy (Kim et al., 2008). Three other heterozygous frame shift CAV1 mutations were found later in patients with similar phenotype: c.88delC and p.I134fsdelA-X137 in (Cao et al., 2008) and p.Phe160X in (Schrauwen et al., 2015). Three PTRF (cavin-1) mutations have also been found in patients with general lipodystrophy: c.696_697insC and c.525delG in (Hayashi et al., 2009), and c.947delA in (Ardisson et al., 2013). These studies, together with others, confirm the central role of caveolae in lipid homeostasis.

2.4.2 Vascular and pulmonary dysfunction

Cav1 and cavin-1 deficient mice also experience vascular dysfunctions. Indeed, abnormal artery vasodilatation and relaxation as well as impaired myogenic tone were observed in Cav1 KO mice (Drab, 2001; Razani et al., 2001). In these models, increased eNOS activity was shown to be responsible for these defects, confirming previous report of eNOS inhibition by Cav1 (see chapter 2.2.3). Calcium could also be involved in the contractility phenotype as it has been shown that calcium waves enter the cell via regions enriched in caveolae (Isshiki et al., 1998). Many other signaling pathways specific to endothelial cells are actually associated with caveolae (reviewed in Frank et al., 2003), including the VEGF (Vascular endothelial growth factor) pathway. This could partially explain the decreased angiogenesis observed in Cav1 KO mice (Woodman et al., 2003). On the contrary, high levels of Cav1 expression have been associated with atherosclerosis (Fernández-Hernando et al., 2010). In the particular case where Cav1 is overexpressed (OE-Cav1) specifically in mouse endothelial cells, cell proliferation and migration, normally necessary to reseal wound and avoid plaque accumulation, are decreased. NO production was also decreased, probably as a consequence of the enhanced eNOS inhibition by OE-Cav1. This could accelerate atherosclerosis development, as observed in eNOS KO mice (Kuhlencordt et al., 2001). Finally, Cav1 overexpression led to increased VCAM (vascular cell adhesion molecule) expression, involved in the recruitment and differentiation of monocytes into macrophages, which participate to inflammation.

In addition to vascular dysfunction, Cav1 mice exhibit also lung abnormalities, consisting in thickened alveolar septa due to cell hyperproliferation (Razani et al., 2001). It is also accompanied by increased fibrillar deposit, that could lead to the initiation of pulmonary fibrosis (Drab, 2001). In addition, pulmonary hypertension was observed in patients

bearing either the F160X or the P158PfsX22 mutations (Austin et al., 2012; Han et al., 2016; Schrauwen et al., 2015).

2.4.3 Cancer

Many studies have long investigated the link between caveolae and cancer but no consensus has been reached yet. Most of the studies focus on the implication of Cav1, and it is yet not well understood whether Cav1 is a tumor suppressor or an oncogene (Lamaze & Torrino, 2015; Martinez-Outschoorn et al., 2015).

Because the CAV1 gene is often deleted in human cancer such as squamous cell carcinomas, head and neck, and prostate cancer, ovarian adenocarcinomas, colon carcinomas and breast cancers, Cav1 was rather qualified as a tumor suppressor (Engelman et al., 1998). These observations were supported by the fact that 3T3 fibroblasts depleted for Cav1 could form tumor in nude mice through hyperactivation of the MAPK pathway (Galbiati et al., 1998). On the contrary, pancreatic carcinoma cells overexpressing Cav1 had reduced tumor formation *in vivo*. In this case, Cav1 overexpression induced inhibition of MAPK signaling, leading to reduced cell growth and anchorage-independent growth (Han et al., 2009). Furthermore, the P132L mutant of Cav1, retained in the Golgi complex and acting in a dominant negative fashion, leads to increased EGF and MAPK pathway activation and have been observed in some cancers (Hayashi et al., 2001). But the defined role of the P132L mutant in cancer is to date still controversed (Lacroix-Triki et al., 2010). Cav1 can also restore K-ras induced senescence that is normally lost in A549 and H460 lung cancer cells (Volonte et al., 2017). Surprisingly, Cav1 KO mice don't develop spontaneous tumors (Razani et al., 2001) but have enhanced sensitivity to chemical carcinogen induced tumor formation (Capozza et al., 2003).

On the other hand, Cav1 expression has also been shown to be upregulated in bladder, esophagus, thyroid and prostate carcinomas (reviewed in Williams & Lisanti, 2005),

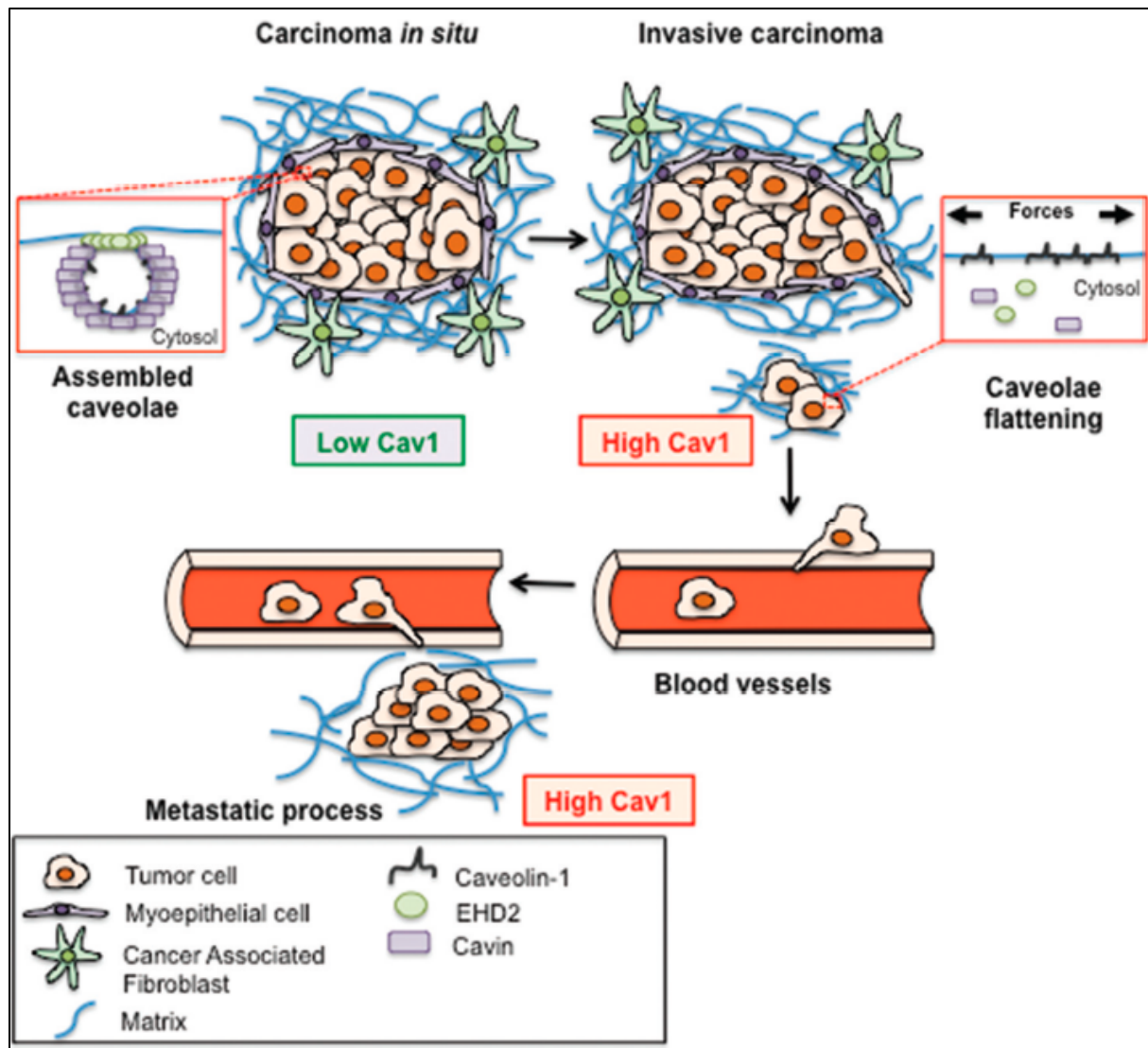


Figure 17. Potential role of caveolae in tumor progression.

Tumors are often characterized by enhanced rigidity and stiffness and recent evidence shows that tumor progression is associated with alterations in tissue and cell mechanics. Caveolin-1 (Cav1), the main constituent of caveolae, is clearly involved in tumor progression. A biphasic expression pattern could be correlated with distinct Cav1 functions. It was shown that Cav1 expression is low during the first stage of tumor progression, however, Cav1 is overexpressed during the advanced cancer phases, including metastatic process. Thus, Cav1 would act as a tumor suppressor at early stage of transformation and tumor progression while it would play an oncogenic role inducing migration and metastasis at later stages. Our hypothesis is that the dual role of Cav1 in tumor progression may be explained by their recently discovered new function as mechanosensors that adapt the cell response to mechanical forces. Thus, in in situ carcinoma, when proliferative tumor cells become confined by the basal membrane, functional caveolae respond as mechanosensors with cycles of caveolae disassembly/reassembly induced by external forces. In invasive carcinoma, tumor cells break down the basal membrane and invade the extracellular matrix. Tumor cells are thus exposed to mechanical forces generated by the extracellular matrix and tissue stiffness. Increased mechanical environment may overwhelm and alter the functional cycle of caveolae disassembly/reassembly. This in turn may impair the caveolae mechanical response and Cav1 dependent mechanosignaling thereby promoting migration and metastasis formation. (Lamaze & Torino, 2015)

suggesting an oncogene role. This is supported by studies showing that Cav1 promotes tumor cell migration and invasion through rac1 (Ras-related C3 botulinum toxin substrate 1) activation both in metastatic cell lines and in mice injected with a melanoma cell line (Díaz et al., 2014; Lobos-González et al., 2013). Cav1 can also promote migration through the regulation of focal adhesion dynamics upon Cav1 Tyr14 phosphorylation in metastatic cell lines (Joshi et al., 2008). In pancreatic cancer, Cav1 expression is correlated with increased tumor grade and seems to increase cell proliferation, invasion and migration both *in vitro* and *in vivo* (Chatterjee et al., 2015).

Interestingly, injection of melanoma cells overexpressing Cav1 do not induce spontaneous tumor formation but enhanced metastasis (Lobos-González et al., 2013), suggesting that Cav1 expression could be either a tumor suppressor or an oncogene depending of the stage of the tumor. Indeed, Cav1 can be overexpressed in metastatic prostate tumor compared to primary prostate tumor (Yang et al., 1998).

Cav1 expression in cells present in the microenvironment of the tumor, such as cancer associated fibroblasts (CAF), can also influence cancer progression. Cav1 expression in breast cancer CAFs is associated with low survival. In CAFs, Cav1 controls the localization and inhibits P190 activity, leading to activation of rac. This induces cell contractility, allowing the remodeling and the increased stiffness of the ECM, which is crucial to facilitate cancer cell invasion (Goetz et al., 2011). Cav1 involvement in tumor progression is summarized in **Figure 17**.

2.4.4 Muscle diseases

One other major phenotype associated with cavin-1 deficiency is abnormalities in muscle tissue, either cardiac or skeletal (Ardissone et al., 2013; Ogata et al., 2014; Taniguchi et al., 2016). This is not surprising given the fact that Cavin-1 is necessary for caveolae formation muscle cells, together with the muscle specific isoform, Cav3. The role of

caveolae and Cav3 in muscle diseases, which is the focus of my PhD work, is more extensively detailed in the next chapter.

Phenotype	Symptoms	Muscle biopsy features	Mutation	Reference(s)
LGMD1-C	<p>Restricted to limb muscle</p> <p>Myalgia (muscle pain)</p> <p>Mild to moderate muscle Weakness</p> <p>Calf hypertrophy</p> <p>Muscle cramps after exercise</p> <p>High serum CK levels</p>	<p>Scattered necrotic fibers</p> <p>Increased connective tissue</p> <p>Variability of muscle fiber size</p> <p>Degenerating/regenerating muscle fibers</p> <p>Increased central nuclei</p>	R26Q	(Figarella-Branger et al., 2003)
			D27E	(Fischer et al., 2003)
			V43E	(Sugie et al., 2004)
			A45T	(Herrmann et al., 2000)
			T63P	(Matsuda et al., 2001)
			ΔTFT 63-65	(Minetti et al., 2002)
			S60R	(Fulizio et al., 2005)
			A92T	(Kubisch et al., 2003)
			P104L	(Minetti et al., 2002)
			T77M	(Traverso et al., 2008)
			F96 del	(Cagliani et al., 2003)
			R125H	(De Paula et al., 2001)
HCK	<p>High serum CK concentrations</p> <p>No sign of muscle disease</p>	<p>Mild variability of muscle fiber size</p>	R26Q	(Carbone et al., 2000)
			P28L	(Merlini et al., 2002)
			N33K	(Fulizio et al., 2005)
			S60R	(Fulizio et al., 2005)
			T77K	(Traverso et al., 2008)
			T77M	(Reijneveld et al., 2006)
			F96 del	(Cagliani et al., 2003)
RMD	<p>Increased muscle irritability, 3 means :</p> <p>1) Percussion induced rapid contraction (PIRC)</p> <p>2) Percussion-induced muscle mounding (PIMM)</p> <p>3) Rippling muscle</p> <p>Muscle hypertrophy</p>	<p>Variability of muscle fiber size</p> <p>Increased central nuclei</p>	R26Q	(Vorgerd et al., 2001)
			D27E	(Fischer et al., 2003)
			P28T	(Van Den Bergh et al., 2004)
			A45T	(Betz et al., 2001)
			E46K	(Madrid et al. 2005)
			W70X	(Ueyama et al., 2007)
			A92T	(Kubisch et al., 2003)
			P104L	(Betz et al., 2001)
			F96 del	(Cagliani et al., 2003)
R125H	(De Paula et al., 2001)			
DM	<p>Restricted to distal muscle</p> <p>Muscle weakness</p> <p>High serum CK concentrations</p>	<p>Mild variability of muscle fiber size</p> <p>Increased central nuclei</p> <p>Increased connective tissue</p>	D27E	(Fischer et al., 2003)
			N33K	(Fulizio et al., 2005)

Table 1. Cav3 mutations and associated caveolinopathies

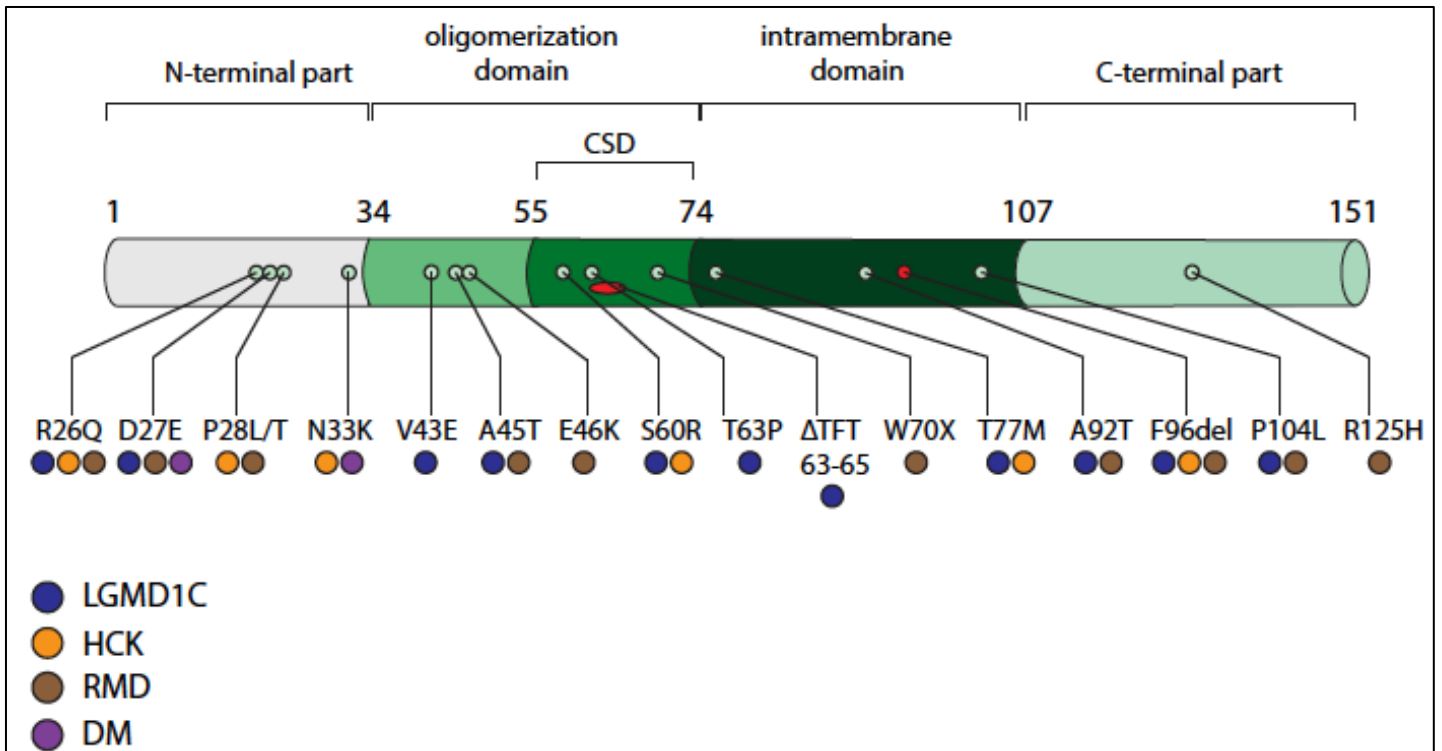


Figure 18. Muscle disorders associated caveolin-3 mutations.

Schematic representation of caveolin-3 (Cav3) mutations localization along the Cav3 protein. Each mutation can be associated with one or more Cav3 related musculopathies that are classified as four different phenotypes: limb-girdle muscular dystrophy 1C (LGMD1C), hyperCKemia (HCK), rippling muscle disease (RMD) and distal myopathy (DM). Based on (Parton & del Pozo, 2013).

3 Cav3 and caveolae in muscle physiology and pathophysiology

3.1 Cav3 mutations and muscle diseases

As mentioned before, caveolae are highly enriched in the muscle tissue, and Cav3 is one of the major players in caveolae biogenesis in muscle cells. Not surprisingly, CAV3 mutations leading to defects in quantity and/or quality of the Cav proteins have been associated with muscle diseases. Caveolinopathies is a group of genetic muscular disorders associated with CAV3 mutations, which gather four different phenotypes in which skeletal muscles are affected: limb girdle muscular dystrophy 1C (LGMD1-C), isolated hyperCKemia (HCK), rippling muscle disease (RMD) and distal myopathy (DM). Symptoms and a non-exhaustive list of Cav3 mutations associated with the different caveolinopathies are summarized in **Table 1**. Among caveolae components, cavin-1 has also been associated with muscle diseases, although in a less defined manner as compared to Cav3. Cavin-1 mutations usually lead to lipodystrophy, but in some cases, patients also experience muscular dystrophy. In most of these patients, symptoms are the same as for CAV3 mutations, consisting in mild muscle pain and weakness along with muscle hypertrophy. Muscle biopsies show variations in muscle fiber size, increased central nuclei and fibrosis (Ardisson et al., 2013; Hayashi et al., 2009; Shastry et al., 2010).

CAV3 mutations are located all along the Cav3 protein and can affect all the domains, although very few mutations have been identified in the C-terminus part (**Figure 18**). Interestingly, one mutation can lead to different phenotypes. For instance, Cav3-R26Q has been found in patients presenting one of each phenotype: LGMD1-C (Figarella-Branger et al., 2003), HCK (Carbone et al., 2000) or RMD (Vorgerd et al., 2001). This mutation can also be found associated with several phenotypes within the same patient,

as found in this family of 14 members, where 12 members had all the three phenotypes overlapping (Carbone et al., 2000). Surprisingly, the observed molecular phenotype of CAV3 mutations is the same for almost all described mutations. Very often, Cav3 is expressed at lower level, and in 95% of the cases, it is retained at the Golgi complex, with a resulting loss of Cav3 and hence caveolae at the PM.

Cavin-1 mutations usually lead to a strong decrease in the expression of the protein, which results in the absence of caveolae and their mislocalization or a decreased expression of Cav3, inducing a loss of caveolae structures at the plasma membrane (Ardissone et al., 2013; Hayashi et al., 2009). These diseases are thus due to either a Cav3 or a cavin-1 loss of function.

The generation of KO mice and the identification of multiple CAV3 and cavin-1 mutations will allow to better define the role of Cav3 and caveolae functions in the skeletal muscle, which is key for understanding their implication in muscle diseases.

3.2 Cav3 and caveolae in muscles

3.2.1 Myofiber integrity

3.2.1.1 Mechanoprotection

Mechanoprotection is extremely important for the integrity of tissues undergoing constant mechanical stress, like the muscle tissue, and caveolae seem to play a major role in this process. Confirming the initial observations made in endothelial and muscle cells (Sinha et al., 2011), it has been shown that in the muscle fiber, caveolae flatten upon mechanical stress, with a preferential impact on the rosette structures that are also disassembled (Lo et al., 2015). The unfolding of caveolae structures by providing more membrane area contributes to the buffering of membrane tension increases that occur during mechanical stress in myotubes, as myotubes expressing the P28L mutation, which results in Cav3 retention in the Golgi complex, fail to do so (Sinha et al., 2011). Furthermore, myofibers lacking cavin-1, and thus caveolae, are more prone to membrane

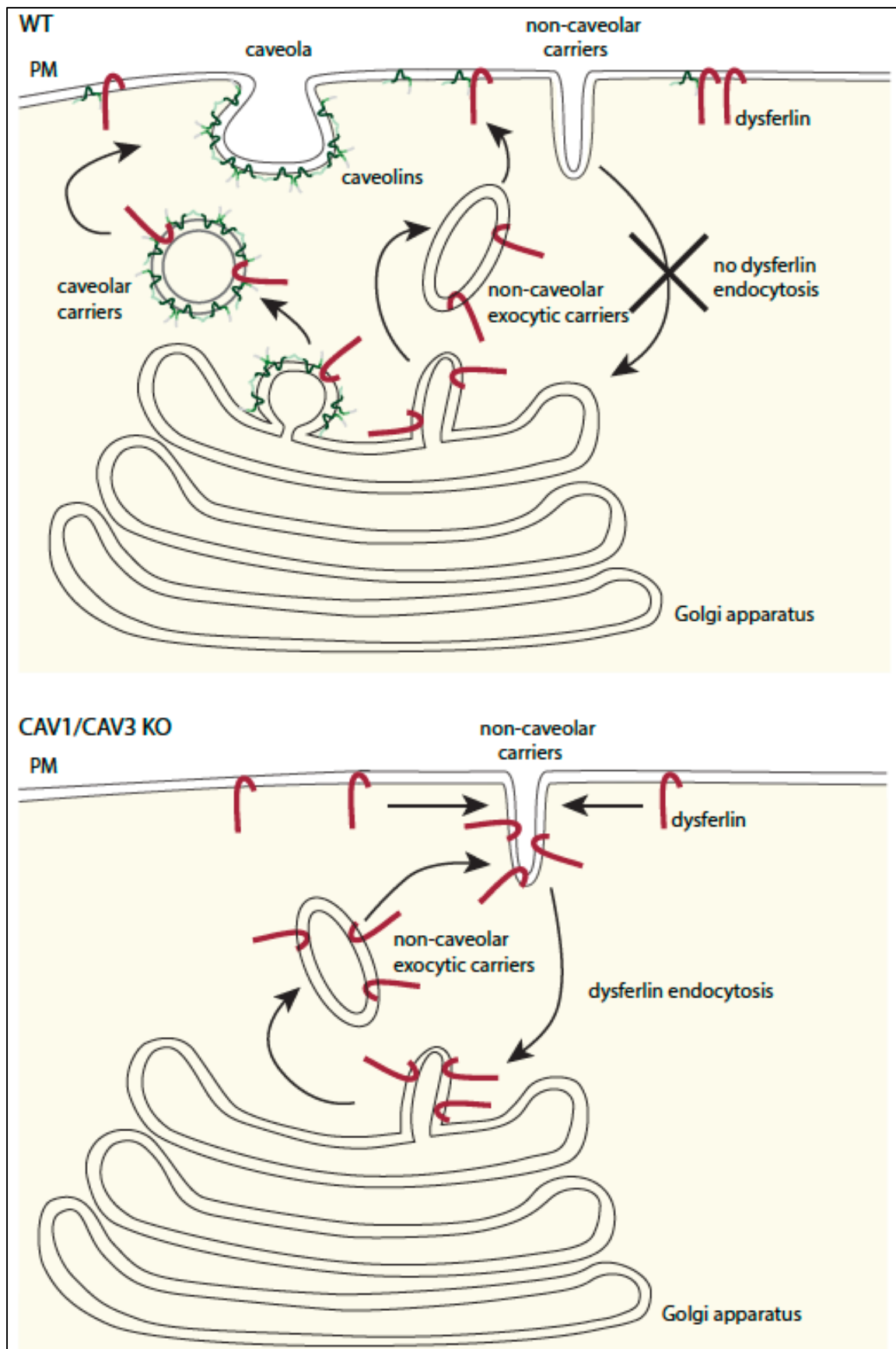


Figure 19. Model for caveolin dependent dysferlin trafficking.

In WT cells, dysferlin exit from the Golgi complex may take place via caveolar and non-caveolar exocytic carriers. Once at the plasma membrane, dysferlin endocytosis is inhibited by dysferlin association with caveolin.

In Cav1 or Cav3 deficient cells (Cav1/Cav3 KO), dysferlin exit from the Golgi complex only relies on non-caveolar exocytic carriers. But dysferlin is rapidly endocytosed as caveolin absence enables dysferlin retention at the plasma membrane. Based on (Hernandez-Deviez et al., 2008).

rupture upon mechanical stress, which is also the case in muscle fibers of zebrafish bearing the R26Q mutation when they are put in viscous media (Lo et al., 2015). This defect could partially explain the degenerating/regenerating fibers that are often observed in muscle disease and that can result from impaired membrane integrity followed by tissue regeneration.

3.2.1.2 Membrane repair

Caveolins have been shown to be associated with dysferlin, which, as mentioned before, is a key protein for membrane repair. Cav3 and dysferlin interact in muscle cells and are located together at the sarcolemma or near T-tubules (Hernández-Deviez et al., 2006; Matsuda et al., 2001). Furthermore, the absence of caveolae led to a highly decreased plasma membrane localization of dysferlin, which was mainly mislocated in internal punctuated structures (Hernández-Deviez et al., 2006). This abnormal localization is the result of a decreased delivery of dysferlin to the PM, coupled with enhanced endocytosis of dysferlin (Hernandez-Deviez et al., 2008). Caveolae are thus important for dysferlin delivery to, and proper localization and retention at the PM (**Figure 19**). MG53, the third protein forming the complex with Cav3 and dysferlin that is needed for membrane repair (see chapter 1.2.3) is also affected in C2C12 differentiated mouse myotubes expressing Cav3-P104L. The Cav3-P104L mutant also induced the retention of MG53 in the Golgi complex, leading to deficient recruitment of MG53 containing vesicles resulting in lack of membrane repair after injury (Cai et al., 2009).

Another mechanism of caveolae dependent membrane resealing was described earlier in chapter 2.3.4, consisting in caveolae cluster formation at the site of injury, followed by caveolae mediated endocytosis resealing the wound. Interestingly, this mechanism seems to occur also in muscle fibers (Corrotte et al., 2013).

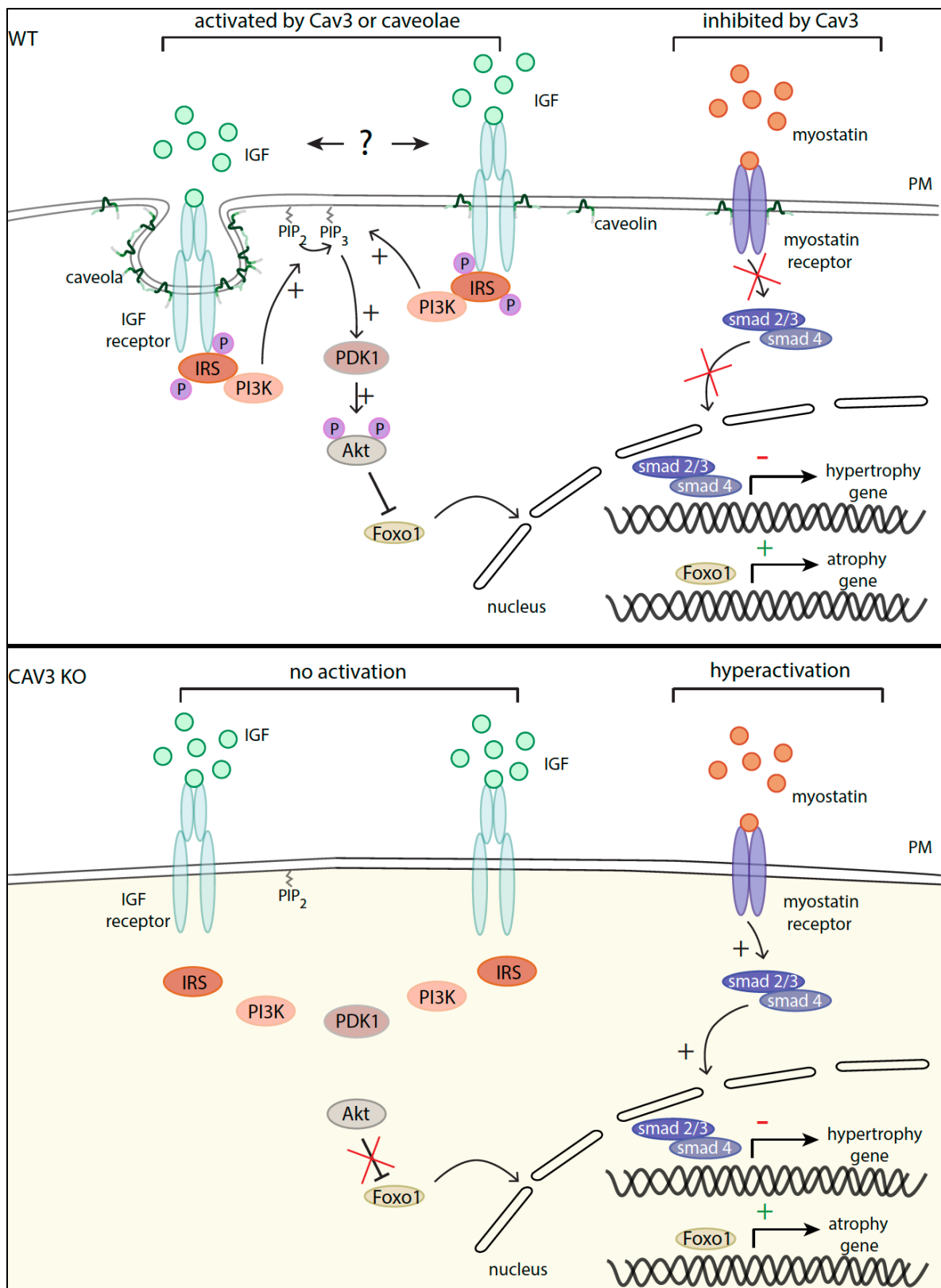


Figure 20. Caveolin-3 promotes muscle growth.

In WT cells, upon insulin-like growth factor (IGF) stimulation, the activation of the associated pathway requires the interaction between Cav3 and the IGF receptor. Activation of the receptor is followed by the recruitment and activation of PI3K. This kinase converts PIP₂ to PIP₃, which serves as a platform for the recruitment of PDK1 and Akt. Active Akt inhibits Foxo1, a transcription factor that positively regulates the expression of atrophy-related genes. In parallel, Cav3 also interacts with the myostatin receptor, inhibiting its activity. This results in the lack of activation of smad2/3 and smad4, which normally regulate negatively the expression of hypertrophy-related genes. Thus, in WT cells, Cav3 contributes to muscle growth.

In Cav3 deficient cells (Cav3 KO), The IGF/Akt pathway is inhibited, leading to the abolished inhibition of Foxo1 activity. In parallel, the myostatin pathway is no longer inhibited. Thus, lack of Cav3 contributes to muscle atrophy.

3.2.1.3 Myoblast fusion, muscle atrophy and muscle hypertrophy

Myoblast activation, proliferation, differentiation and fusion are key for muscle biogenesis and regeneration after injury. Cav3 has first been described as a positive regulator of myoblast fusion, as its depletion in C2C12 myoblasts leads to lack of myotube formation (Galbiati et al., 1999). The same phenotype was observed in C2C12 myoblasts transfected with a LGMD-1C associated CAV3 mutation (P104L). The mutated myoblasts present lower levels of Akt and p38 MAP kinase basal activation, leading to defects in myoblast differentiation and fusion (Stoppani et al., 2011). Indeed, it has been shown that activation of these pathways are required for myoblast differentiation (Li et al., 2000). Another pathway involved in the regulation of myoblast differentiation is the myostatin pathway. Myostatin is a muscle specific member of the TGF family that is a key player in the inhibition of myoblast differentiation, as it negatively controls the expression of myoblast differentiation associated genes (Joulia et al., 2003). Interestingly, Cav3 has been shown to interact with the myostatin receptor, probably through CSD-CBM interaction, as this motif has been identified in the cytosolic tail of the receptor (Ohsawa et al., 2006). This interaction results in decreased activation of the myostatin pathway, suggesting that Cav3 is a negative regulator of the pathway. In accordance, myofibers from transgenic mice bearing the Cav3-P104L mutation or from patients carrying two different cavin-1 mutations (c.696_697insC and c.525delG) present an hyperactivation of the myostatin pathway (Hayashi et al., 2009; Ohsawa et al., 2006). The absence of Cav3 is thus associated with defect in myoblast differentiation and fusion through both decreased Akt activity and enhanced myostatin activity and resulting in muscle atrophy (**Figure 20**).

Another study showed on the contrary that transgenic mice overexpressing Cav3 feature inhibition of myoblast fusion, while mice deficient for Cav3 showed increased myoblast fusion, leading to aberrant muscle fiber size. In this case, Cav3 would act through the inhibition of M-Cadherin, a protein targeted to caveolae that is involved in cell fusion

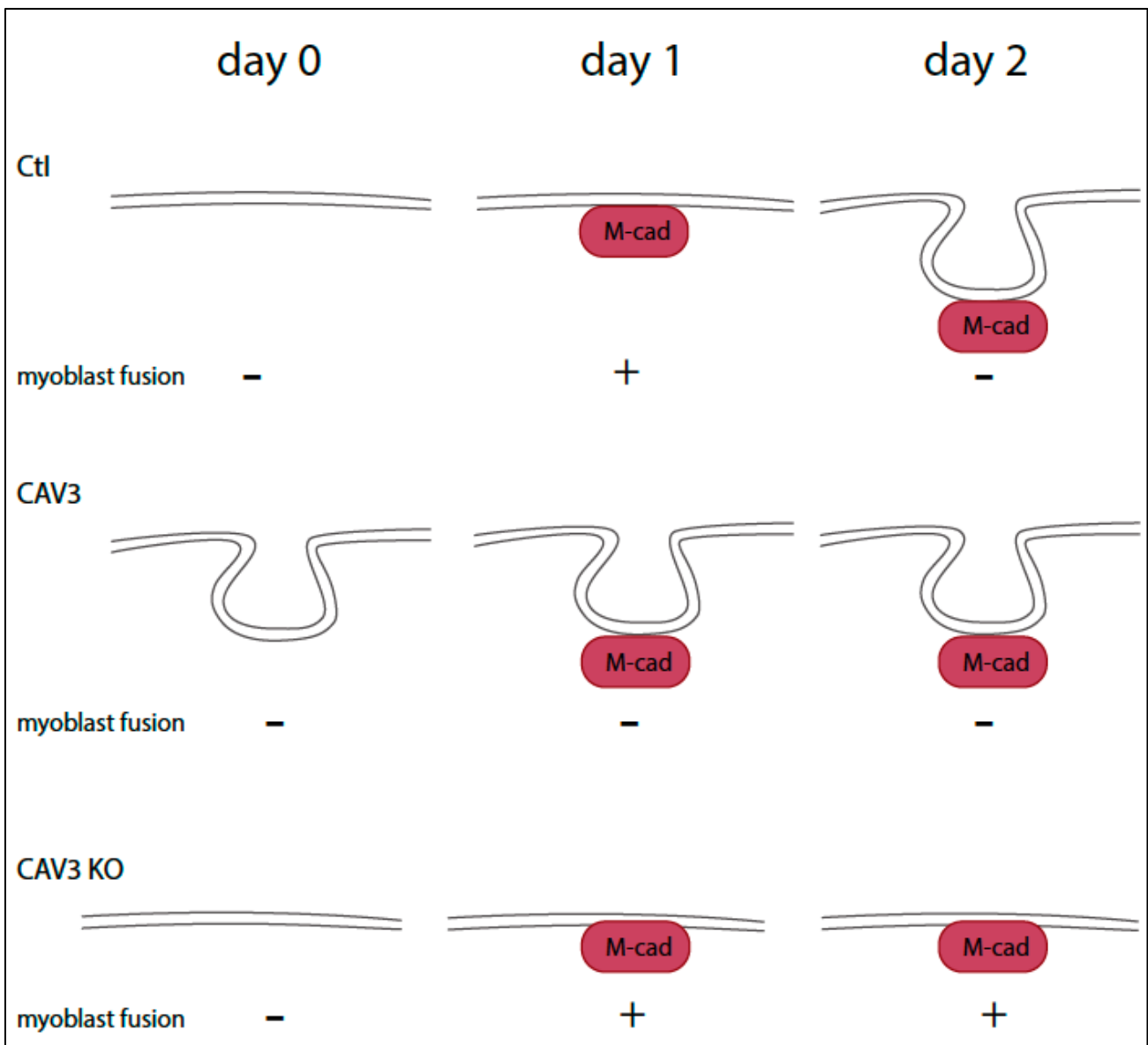


Figure 21. Modulation of myoblast fusion by caveolin-3.

M-cadherin stimulates myoblast fusion in normal control skeletal muscle cells (Ctl) as long as caveolin-3 is not expressed. After 2 days of differentiation, upregulation of caveolin-3 leads to sequestration of M-cadherin into caveolae membranes and, as a consequence, inhibition of myoblast fusion. Caveolin-3 is always expressed in caveolin-3 transgenic cells (Cav-3) and M-cadherin is constantly enriched into caveolae. As a result, myoblast fusion is inhibited by overexpression of caveolin-3. In contrast, inhibition of myoblast fusion after 2 days of differentiation does not occur in caveolin-3 null myotubes (Cav3 KO), which do not express caveolin-3. (Volonte et al., 2003)

(Volonte et al., 2003). In this case, Cav3 deficiency leads to muscle hypertrophy (**Figure 21**). Both atrophy and hypertrophy are observed in muscle disease, although LGMD1-C and RMD has so far mainly be associated with hypertrophy.

3.2.2 Myofiber function

Caveolae and Cav3 have been described to be associated with several key muscle structures such as T-tubules or the costamere, and with signaling pathways important for muscle function, that will be described in the present chapter.

3.2.2.1 Triad formation and function

Electron microscopy performed on skeletal muscle cells isolated from a chick embryo allowed for the first time the visualization of structures looking like clusters of caveolae in the chicken muscle. It was proposed that these structures were precursors of T-tubules (Ishikawa, 1968). Cav3 and caveolae were indeed found to be associated with developing T-tubules in mice embryos but this association was transient. In adult mice muscle tissue, Cav3 is no longer associated with T-tubule but is rather located at the sarcolemma (Parton et al., 1997). Other data in Cav3, cavin-1 or cavin-4 deficient mice or zebrafish also show abnormal T-tubule organization (Galbiati et al., 2001; Housley et al., 2016; Lo et al., 2015). This phenotype could be relevant in the context of muscle diseases, since skeletal muscles from a LGMD-1C patient showed T-tubule disorganization associated with large vacuolar structures below the sarcolemma, probably resulting from T-tubule abnormal proliferation (Minetti et al., 2002). The same kind of vacuoles were found in the muscle of RMD and DM patients (Fischer et al., 2003). T-tubules have a different lipid composition than the rest of the sarcolemma, and are particularly enriched in cholesterol (Horgan & Kuypers, 1987). It has thus been proposed that caveolae would bring a particular set of lipids necessary for T-tubule formation, but

also to allow the proper organization of T-tubule associated proteins such as the DHPR (see chapter 1.3.2). Indeed, the DHPR was more diffusely localized in skeletal muscle fibers of Cav3 deficient mice compared to WT (wild type) (Ferruccio Galbiati et al., 2001). Furthermore, Cav3 is able to interact with DHPR and RyR1, with which it forms two kind of complexes linking T-tubule with the sarcoplasmic reticulum. One is composed of Cav3, DHPR and RyR1 and the other with Cav3, RyR1 and two triadins, Trisk 51 and Trisk 95 (Vassilopoulos et al., 2010). This is supported by the lack of colocalization between DHPR and RyR1 in myotubes carrying two different RMD associated Cav3 mutations, which leads to a reduced efficiency of the excitation-contraction coupling (Ullrich et al., 2011). Finally SERCA pumps were shown to be co-fractionated with caveolae in sarcoplasmic reticulum membranes (Li et al., 2006), suggesting that Cav3 is tightly associated with calcium release in myofibers.

3.2.2.2 Costamere formation

At the level of the costamere, Cav3 has been shown to be co-fractionated with members of the DGC complex (Song et al., 1996), and more precisely through the interaction between the Cav3 WW domain and the PPXY motif located in the C-terminal tail of β -dystroglycan (see chapter 1.3.2). This is the same domain interacting with dystrophin, implying that Cav3 can be in competition with dystrophin for β -dystroglycan binding (Sotgia et al., 2000). This may explain why the overexpression of Cav3 induces the destabilization and degradation of the DGC complex, which leads to major defects in membrane integrity (Galbiati et al., 2000). In CAV3 KO mice, the DGC complex is expressed at normal levels but is no longer co-fractionated with lipid raft, showing that Cav3 is necessary for providing a specific lipid environment to the DGC complex (Galbiati et al., 2001). Surprisingly, in muscle fibers from patients with RMD or LGMD-1C, α -sarcoglycan was no longer expressed, causing the dissociation of the DGC complex and the lack of association to the ECM (Herrmann et al., 2000; Vorgerd et al., 2001).

3.2.2.4 Calcium, NO and muscle contraction

Calcium is a key player in muscle contraction and in addition to the Ca^{2+} released from the SR, it can also be imported from outside via calcium channels. In skeletal muscle cells, TRPC1 (transient receptor potential channel 1) is a calcium channel important for Ca^{2+} influx during repeated contractions. Indeed, mice deficient for TRCP1 exhibit a progressive loss of force and a decrease in endurance activity (Zanou et al., 2009). It has been shown that Cav3 play a role in the targeting of TRPC1 at the plasma membrane and thus in Ca^{2+} entry (Gervasio et al., 2008). Cav3 could also play a role in the activation of the channel, since stretch induced Src kinase activation, which leads to activation of TRPC1, was increased in Cav3 overexpressing myoblasts (Gervásio et al., 2011).

Nitric oxide (NO) is also another important regulator of muscle contraction. Indeed, NO production decreases the mechanical force induced during contraction through the activation of cGMP and inhibition of RYR1 opening (Meszaros et al., 1996; Stamler & Meissner, 2001). NO is also involved in other myofiber functions, such as glucose uptake and mitochondria respiration (Reid, 1998; Stamler & Meissner, 2001). NO is produced by NOS, that exists under three isoforms, nNOS, eNOS, and iNOS (neuronal-, endothelial-, and inducible- nitric oxide synthase). All three isoforms are expressed in skeletal muscle but nNOS, located at the sarcolemma, is the one producing the majority of NO found in myofibers. nNOS binds both α -syntrophin, a member of the DGC complex, and its expression and localization are impaired in myotubes lacking dystrophin (Brenman et al., 1995). nNOS is also tightly linked to Cav3, as it has been shown to interacts with the Cav3 scaffolding domain leading to the inhibition of its activity (Garcia-Cardena et al., 1997; Venema et al., 1997). Supporting this, increased nNOS activity was observed in transgenic mice carrying the P104L mutation (Sunada et al., 2001), but also in a patients carrying two different cavin-1 mutations (c.696_697insC and c.525delG), situation in

which Cav3 is mostly cytoplasmic and caveolae structures are absent at the plasma membrane (Hayashi et al., 2009). It is worth mentioning that investigating nNOS activity in Cav3 associated muscular diseases could be difficult as CAV3 mutations can also induce dissociation of the DGC complex, leading to the absence of nNOS, as reported in muscle fibers from patients with LGMD1-C (Herrmann et al., 2000).

3.2.2.4 Glucose metabolism

The insulin pathway, which is a key pathway for glucose uptake and glycogen synthesis in skeletal muscles (see chapter 1.3.3.1 *The glucose pathway in muscle*), is tightly associated to caveolae. Indeed, Cav3 has been shown to enhance insulin response when it is overexpressed, and the use of CSD mimicking peptide is sufficient to recapitulate this effect *in vitro* (Yamamoto et al., 1998). This was confirmed by a decreased association to IRS-1 (insulin receptor substrate 1) and PI3K (phosphoinositide 3-kinase), resulting in lower PKB/Akt (protein kinase B) phosphorylation and activation in skeletal myofibers from CAV3 KO mice. After insulin stimulation, the recruitment of GLUT4 to the plasma membrane follows a two-step process, both depending on caveolae. Insulin stimulation dependent PI3K/Akt activation allows the trafficking of GLUT4/flotilin-1 containing vesicles away from the periphery of the nucleus. Then, Cav3 and the insulin receptor containing endocytic vesicles move from the sarcolemma to the cytoplasm, where they transiently interact with GLUT4/flotilin vesicles. The insulin receptor is translocated to GLUT4/flotilin vesicles, which induces the recruitment and activation of the Cbl/C3G/TC10 pathway and the further movement of vesicles to the plasma membrane (Fecchi et al., 2006). It is thus not surprising that Cav3 deficient mice exhibit an insulin resistant phenotype (Oshikawa et al., 2004). Supporting these findings, Cav3 overexpression leads to increased Akt activation, even without insulin stimulation, and to increased recruitment of GLUT4 at the plasma membrane and glucose uptake. Furthermore, Akt activation promotes the growth and proliferation of muscle cells

overexpressing Cav3. Surprisingly, glycogen levels were unchanged in Cav3 overexpressing myofibers in comparison with normal myofibers, suggesting that in this case, it might involve a pathway independent of insulin (Shang et al., 2017). Recently, the Cav3-P104L mutation was also shown to cause glucose metabolism disorders in skeletal muscle. Indeed, C2C12 myotubes transfected with Cav3-P104L, and differentiated in high glucose medium, also display glucose metabolism disorders. In these myotubes, Akt activation was decreased, leading to a defect in GLUT4 containing vesicles to the plasma membrane, resulting in low glucose uptake and glycogen synthesis (Deng et al., 2017). Glucose metabolism disorders can also be due to the regulation of phosphofructokinase (PFK) by Cav3. PFK is the key enzyme for glycolysis, and it has been shown to interact with Cav3 upon high glucose levels in the extracellular environment (Scherer & Lisanti, 1997). Cav3 allows the targeting of PFK at the plasma membrane and its localization in Cav3-rich domains. Indeed, both myofibers from Cav3 deficient mice and cells expressing LGMD-1C associated Cav3 mutations present an intracellular localization of PFK, but also degradation of this enzyme in the case of Cav3 mutant (Sotgia, et al., 2003). Although it has not been established whether the mislocalization of PFK can directly affect glycolysis, it can be hypothesized that Cav3 allows the concentration and stability of PFK tetramers, which is the fully active structure of the enzyme (Sola-penn et al., 2010).

Caveolae play thus a central role in different aspects of the skeletal muscle physiology. They are important for muscle formation and size regulation, as Cav3 and caveolae are key for myoblast differentiation and fusion, as it has been shown that deregulation of Cav3 expression can both induce atrophy and hypertrophy. Caveolae play also a major role in mechanoprotection and are involved in both membrane integrity during mechanical stress and repair following injury. Caveolae are also involved in muscle function, as they have a central role in the regulation of contraction through formation of

the T-tubule or the regulation of contraction-associated pathways. Finally, caveolae also participates to glucose homeostasis by tightly regulating the insulin pathway.

- RESULTS -

4 Lack of functional caveolae in Cav3 P28L and Cav3 R26Q human dystrophic myotubes results in deficient mechanoprotection and IL6/STAT3 mechanosignaling.

4.1 Objectives and summary

A few years ago, our laboratory discovered a new role for caveolae as mechanosensors that control cell membrane tension homeostasis under mechanical stress: caveolae unfold to provide additional membrane, which buffers membrane tension increase and confers mechanoprotection. The membrane reservoir property of caveolae was also demonstrated in the skeletal muscle, as the Cav3 P28L mutation, which abnormally retain Cav3 in the Golgi complex, led to impaired membrane tension buffering and mechanoprotection in human myotubes upon mechanical stress (Sinha et al., 2011). This new aspect of caveolae function was confirmed by data in zebrafish where the Cav3 R26Q mutation was associated with higher muscle damage upon mechanical stress (Lo et al., 2015). Importantly, our laboratory proposed the hypothesis that caveolae unfolding could be also be of importance in the regulation of certain signaling pathways through the mechanical-dependent release of free i.e. non caveolar caveolins in the plasma membrane (Nassoy & Lamaze, 2012). Giving the rising interest on caveolae in the cell mechanical response, my PhD work focused on their specific role in human Cav3 related muscle diseases, a less explored field of investigation.

To do so, we studied human myoblasts that were isolated and immortalized from healthy patients, or bearing the Cav3 P28L or Cav3 R26Q mutation. These myoblasts can differentiate into myotubes, allowing us to investigate the effect of the endogenous expression of Cav3 mutations and in a cell type close to primary muscle cells (Mamchaoui

et al., 2011). The determination of Cav3 protein expression and localization, combined with structural EM analyzes of wild type (WT) and mutant myotubes showed that the retention of Cav3 mutants in the Golgi complex led to a drastic decrease of the amount of caveolae structures at the plasma membrane of mutant myotubes.

We next analyzed how this defect could impact cell membrane tension and integrity upon mechanical stress. Membrane tension measurements, performed using the nanotube pulling technique combined with optical tweezers, revealed a lack of membrane tension buffering in mutant myotubes that were submitted to a hypo-osmotic shock. This translated into failed mechanoprotection in mutant myotubes as demonstrated with a membrane bursting assay.

Considering the possible role of caveolae mechanics in signaling (Nassey & Lamaze, 2012), we next wanted to know whether the interleukin-6 signaling pathway could be differently regulated in mutant and wild type myotubes under rest and upon mechanical stress. Indeed, IL6 has been described as a major cytokine that is secreted by muscle cells during physical exercise (Ostrowski et al., 1998; Steensberg et al., 2002). Furthermore, we hypothesized that the IL6 signaling pathway could be regulated by Cav3 and/or caveolae, as one of its receptor subunit gp130 and the associated JAK kinases bear putative caveolin binding motifs, that could interact with the corresponding caveolin scaffolding domain of Cav3. As a matter of fact, by monitoring the IL6 signaling pathway in WT, Cav3 mutants, and Cav3 depleted myotubes, we found that Cav3 could negatively regulate the IL6 signaling pathway. Furthermore, experiments combining Cav3 depletion and hypo-osmotic shocks in human myotubes revealed that the IL6 signaling pathway was also negatively regulated by mechanical stress in a Cav3-dependent manner.

Finally, we made the interesting discovery that the Cav3 mutant myotubes reproduce the phenotype of Cav3 depletion in WT myotubes. We could also induce the formation of caveolae at the plasma membrane and rescue the mechanoprotection and mechanosignaling functions by expressing WT CAV3 in mutant myotubes. Altogether, our results establish for the first time a general defect of the cell mechano-response in human

Cav3 mutant myotubes, as a consequence of the impossibility to assemble a functional reservoir of caveolae at the plasma membrane and to control IL6 stimulation through the mechanical release of free Cav3.

The detailed methods are described in the next chapter, followed by the detailed results presented in the form of an article in preparation for submission.

4.2 Method

Myoblast differentiation

Myoblast are plated at 80-100% confluency on a 0.01% matrigel coated surface to avoid detachment of future myotubes. The medium is then switched with DMEM high-glucose Glutamax supplemented with 0.1% of insulin for 4 days. Myoblast are thus serum starved, which allows the differentiation. Myotubes are defined by the presence of at least two nuclei in the cell. In all experiments performed, myotubes are 4 days differentiated myotubes. Except indicated differently, cells are grown and differentiated on cell culture treated dishes, plates or flasks.

Immunoblotting

After indicated treatment, cells were lysed in sample buffer (62.5 mM Tris/HCl pH 6.0, 2% v/v SDS, 10% glycerol v/v, 40mM dithiothreitol and 0.03% w/v phenol red). Lysates are loaded on a pre-cast polyacrylamide gel, together with a protein ladder to serve as size reference, and submitted to electrophoresis. Proteins are then transferred on a nitrocellulose membrane using a semi-dry transfer apparatus. The membrane is saturated upon incubation in Tris-buffer saline with 0.1% tween20 (TBST) supplemented with 5% BSA for at least 20 min. The membrane is then incubated with indicated primary antibodies (O/N at 4°C at 1/1000 for all antibodies used) in TBST 5% BSA, washed three times for 5 min with TBST and incubated with indicated horseradish peroxidase- conjugated secondary antibody (1 h at RT at 1/5000 for all antibodies used) in TBST 5% BSA. After three washes of 5 min with TBST, the chemiluminescence signal is revealed using Pierce™ ECL Western Blotting Substrate, SuperSignal West Dura Extended Duration Substrate or SuperSignal West Femto Substrate. Acquisition and quantification were performed with a ChemiDoc MP Imaging System. Protein

levels of expression are quantified by calculating the ratio between the intensity of the signal corresponding to the protein of interest and the one of either tubulin or clathrin heavy chain, which serve as loading control.

Immunofluorescence

Myoblasts are differentiated into myotubes on coverslips coated with 0.01% matrigel. For Cav3, Cav1, MF-20 and GM130 staining, myotubes are fixed with 4% PFA for 10min, quenched in 50 mM NH₄Cl and then permeabilized with 0.2% BSA and 0.05% saponin in PBS for 20 min. Cells are incubated sequentially with indicated primary (dilution: Cav3 1/250, Cav1 1/500, MF-20 1/20 and GM130 1/50) and fluorescence-conjugated secondary antibody (1/500 for all antibodies) in permeabilization buffer for 1h at RT. For pSTAT3 staining, cells are fixed and permeabilized with cold methanol for 15 min at -20°C. After washes with PBS 0.2% BSA, cells are incubated sequentially with indicated primary (pSTAT3 1/75) and fluorescence-conjugated secondary antibody (1/500 for all antibodies) in PBS 0.2% for 1h at RT. Coverslips are then mounted in Fluoromount-G mounting medium supplemented with 2 µg/mL of DAPI and are dried O/N at RT. Acquisition of images are done using a spinning disk microscope.

Electron microscopy

Epon embedding was used to preserve the integrity of cell structures. Myotubes were fixed sequentially for 1 h at RT with 1.25% glutaraldehyde in 0.1 M Na-Cacodylate and then overnight at 4°C. Cells were washed extensively with 0.1 M Na-Cacodylate pH 7.2. Membrane fixation was performed for 1 h at RT with 1% OsO₄ in 0.1 M Na-Cacodylate pH 7.2. Cells were dehydrated by incubation with aqueous solutions of ethanol at increasing concentrations (50, 70, 90, then 100%, each for 10 min at RT). Embedding was finally performed in LX112 resin. Cells

were infiltrated with a 1:1 LX112:ethanol solution, washed with LX112, and embedded overnight at 60°C in LX112 resin. Ultrathin 65 nm sections were sliced using a Leica UCT ultramicrotome and mounted on nickel formvar/carbon-coated grids for observations. Contrast was obtained by incubation of the sections for 10 min in 4% uranyl acetate followed by 1 min in lead citrate. Electron micrographs were acquired on a Tecnai Spirit electron microscope (FEI, Eindhoven, The Netherlands) equipped with a 4k CCD camera (EMSIS GmbH, Münster, Germany). Myotubes were defined by cells containing at least two nuclei and caveolae were defined according to their morphology and size, as it was extensively described in the literature.

Micropatterning

18 mm diameter coverslips are coated with PLL-g-PEG (poly L-lysine and polyethylene glycol) for 30min at RT. Coated coverslips are then washed successively in PBS and water, and dried. Coverslips are then placed on a photo-mask on top of a thin layer of water, and are illuminated with UV to destroy the PLL-g-PEG according to the pattern imposed by the mask, in our case in a shape of lines of 10 μm with a separation of 60 μm between the lines. Coverslips are kept at 4°C.

Force measurements

Myotubes are differentiated on line micropattern coverslips coated with 0.01% matrigel. Plasma membrane tethers were extracted from cells by a concanavalin A coated bead (3 μm in diameter) trapped in optical tweezers. The optical tweezers are made of a 1064 nm laser beam (ytterbium fiber laser, $\lambda = 1064 \text{ nm}$, TEM 00, 5 W, IPG Photonics, Oxford, MA) expanded and steered (optics by Elliot Scientific, Harpenden, UK) in the back focal plane of the microscope objective (Apo-TIRF

100Å~ NA 1.45, Nikon). The whole setup was mounted on a Nikon Eclipse-Ti inverted microscope. The sample was illuminated by transmitted light, and movies were acquired at 10 Hz with an EM-charge-coupled device camera (Andor iXon 897) driven by Micro-Manager. The fine movements and particularly the translational movement necessary to pull the membrane tether were performed using a custom-made stage mounted on a piezoelectric element (P753, Physik Instrumente, Karlsruhe, Germany) driven by a servo controller (E665, Physik Instrumente) and a function generator (Sony Tektronix AFG320).

Calibration was performed using an oscillatory modulation driven by a function generator

and measuring the response of the bead to an oscillatory motion of the stage. We measured $k = 16P \text{ pN}/(\mu\text{m}\cdot\text{W})$, where P is the laser power. This relationship is linear in the laser power range used for the experiments (0.4–2 W). The membrane tether was held at constant length to measure the static force. For measuring membrane tension changes due to hypo-osmotic shock, a first tether was first pulled at 300 mOsm (iso condition). A second tube was pulled on the same cell 5 minutes after diluting the medium with milliQ water to obtain 45 mOsm. The position of the bead used to compute tether forces was detected from the images using a custom ImageJ macro.

Membrane burst assay

Myotubes are differentiated on line micropattern coverslips coated with 0.01% matrigel. Myotubes are incubated in 5 $\mu\text{g}/\text{mL}$ of calcein-AM, a permeant dye that becomes fluorescent once it enters intact cells, and 50 $\mu\text{g}/\text{mL}$ of DAPI to distinguish myotubes from myoblasts, in differentiating medium for 15 min at 37°C in the dark. Medium was then switched back with fresh differentiation medium to wash out the excess of calcein-AM and DAPI. The medium is then switched again with a 30 mOsm hypo-osmotic shock medium obtained after a

dilution of 10% medium and 90% H₂O, supplemented with 2 mg/mL of PI, a non permeant dye that binds to DNA and becomes fluorescent in cells with a damaged membrane. Hypo-osmotic shock is used as a mechanical stress as it induces cell swelling. Immediately after medium switching, pictures are taken every minute for 10 min using a videomicroscope. This experiment allows us to study the membrane integrity of WT and mutant myotubes, as the concomitant loss of calcein-AM and appearance of PI fluorescence in a given myotube indicates a membrane bursting event. Membrane integrity is determined through the quantification of the percentage of myotubes that burst and the mean time of hypo-osmotic shock required for a myotube to burst. This assay allows us also to study membrane repair, as the concomitant calcein-AM retention and the appearance of PI fluorescence in a given myotube indicates a membrane repair event. Membrane repair is determined through the quantification of the percentage of myotubes that burst and have a repaired membrane.

IL6 stimulation

Prior to stimulation, myotubes are incubated in insulin free DMEM medium and are thus starved for 4 h.

STAT3 and pSTAT3 immunoblotting:

For IL6 stimulations done in cells at rest, the medium is switched for the indicated time with DMEM supplemented with 0.2% BSA and 10 ng/mL of human recombinant IL6 at 37°C. For IL6 stimulation done in cells submitted to a hypo-osmotic shock, cells are successively incubated in a 75 mOsm hypo-osmotic shock medium (75% H₂O and 25% DMEM) for 5 min at 37°C and in the same medium supplemented with 10 ng/mL of human recombinant IL6 for 5 min at 37°C. For IL6 stimulation done in cells submitted to cyclic stretch, myoblasts are grown and differentiated on stretchable plates coated with 10 µg/mL fibronectin. The plates

are then placed in a stretch device (Flexcell system, Flexcell international) and were subjected or not to 30 min of cyclic stretch (10% elongation, 0.5Hz). Immediately after stretching, the medium was switched with DMEM with 0.2% BSA and supplemented or not with 10 ng/mL of human recombinant IL6 for 5 min. Immediately after IL6 stimulation, Cells are lysed and samples are analyzed by immunoblotting. For the analysis, pSTAT3 levels were quantified by calculating the ratio between pSTAT3 and STAT3, both normalized to Tubulin signal.

pSTAT3 nuclear translocation in IF:

Myoblasts are grown and differentiated on 12 mm diameter coverslips coated with 0.01% matrigel. Cells are stimulated with 10 ng/mL IL6 as described above for 0min or 15min and were then fixed as described in the section *immunofluorescence* and were analyzed for pSTAT3 staining and sub-cellular localization by immunofluorescence. Quantification of pSTAT3 nuclear translocation specifically in myotubes are done by calculating the ratio between the pSTAT3 fluorescent intensity in the nuclei on the one in the cytoplasm. Nuclei are observed with a DAPI staining, and myotubes are identified as cells containing two or more nuclei.

STAT3 related gene expression assay

Myotubes are starved 4h prior to lysis, and RNA extraction is performed using an extraction kit. To obtain corresponding cDNA, reverse-transcription reaction is performed with 1 µg of RNA per reaction, using high capacity cDNA reverse-transcription kit. Samples are placed in a PCR machine with the following program: 10 min at 25°C, 2 h at 37°C, 5 min at 85°C and 4°C hold. qPCR is then performed on 50 ng of cDNA for a reaction in a total volume of 20 µL using the LightCycler 480 Probes Master kit and Taqman gene expression assays that allow the detection of the following genes: FBXO32, SOCS3, ACTC1 and MYH8. GAPDH serves as a house-keeping gene. Samples are then placed in a

LightCycler 480 instrument qPCR machine with the following program: 10 min at 95°C, 45 cycles of 10 min at 95°C + 30 min at 60°C and 1 min at 72°C, 30 min at 40°C. Relative expression levels were calculated using $\Delta\Delta\text{CT}$ method with fold changes calculated as $2^{-\Delta\Delta\text{CT}}$.

RUSH

HeLa cells are transfected with 500ng of Cav3 WT, Cav3 P28L or Cav3 R26Q RUSH constructs (constructs used: Str-li_SBP-EGFP-Cav3, Str-li_SBP-EGFP-Cav3-P28L and Str-li_SBP-EGFP-Cav3-R26Q), Str coding for streptavidine, li coding for the Invariant chain of the major histocompatibility complex and serves as ER hook, SBP coding for streptavidin binding protein, and EGFP coding for eGFP) using a lipofectamin 3000 kit in 6 well plates. 24 h after transfection, cells are incubated at the indicated times in DMEM supplemented with 40 μM of biotin, and immunofluorescence was performed as described in the section *Immunofluorescence* using a GM130 antibody. Cav3 RUSH protein localization is visualized thanks to its eGFP tag. Acquisition of picture is done using a wide-field epifluorescence microscope.

Co-immunoprecipitation

To obtain enough material, myoblasts grown and differentiated in at least two petri dishes of 10 cm diameter and are lysed with a lysis buffer (TNE 150 mM NaCl with 1% NP40 and 1/1000 of protease inhibitor cocktail). Samples are centrifuged 5 min at 13000 rpm to get rid of membranes and an aliquot of supernatant is kept to serve as input. The rest of the samples are further incubated with 3 μg of Cav3 antibody in the lysate buffer O/N at 4°C under agitation to allow capturing of Cav3 and its interacting partners. GFP antibody is used as a negative control. Samples are then incubated with 25 μL of magnetic

beads coated with protein A for 1 h et 4°C under agitation to allow capturing of the antibody coupled with the protein of interest and its partners. Beads are harvest using a magnetic rack, washed to get rid of unspecific binders and proteins at the surface of the beads are put in the buffer used for immunoblotting. Immunoblotting is performed as described in the section *Immunoblotting*.

4.3 Article

Lack of functional caveolae in Cav3 P28L and Cav3 R26Q human dystrophic myotubes results in deficient mechanoprotection and IL6/STAT3 mechanosignaling.

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Abstract

Caveolin-3 is one of the major components of caveolae in muscle cells. Mutations in the CAV3 gene cause different type of muscle disorders mostly characterized by defects in membrane integrity and repair, deregulation in the expression of certain muscle proteins and deregulation of muscle associated signaling pathways. We show here that the lack of functional caveolae at the plasma membrane of myotubes derived from patients bearing the CAV3 P28L and R26Q mutations results in an abnormal mechanoresponse. Mutant myotubes can no longer buffer the increase of membrane tension induced by mechanical stress and present an hyperactivation of the IL6/STAT3 signaling pathway both at rest and under mechanical stress. The lack of mechanical control of the IL6/STAT3 signaling pathway by caveolae leads to a chronic activation of the pathway and a higher expression

of muscle specific genes. The defects in mechanosensing and mechanosignaling observed in mutant myotubes could be reversed by reassembling a functional pool of caveolae through re-expression of WT Cav3. Our findings bring more mechanistic insight into Cav3 associated muscle disorders by showing a general defect in the mechanoresponse of human myotubes expressing the CAV3 P28L and R26Q mutations.

Introduction

Caveolae are cup-shaped plasma membrane invaginations that were first observed in the 50's by Palade and Yamada on electron micrographs from vascular and gall bladder tissues (Palade, 1953, Yamada, 1955). Caveolae present a specific protein signature involving two main families of proteins, caveolins (caveolin-1, -2 and -3), and cavins (cavin-1, -2, -3 and -4) (Aboulaich et al., 2004; Hansen, Howard, & Nichols, 2011; Hill et al., 2008; Morén et al., 2012; Rothberg et al., 1992; Scherer et al., 1996; Way & Parton, 1995). Caveolins and cavins are expressed in almost every type of cells, except for caveolin-3 (Cav3) and cavin-4, which are expressed only in smooth and striated muscle cells (Tagawa et al., 2008; Way & Parton, 1995). Similarly to Cav1 in non muscle cells, Cav3 is necessary for the formation of caveolae at the plasma membrane of muscle cells (Minetti et al., 2002).

Caveolae have long been associated with several important cellular functions including endocytosis, lipid metabolism and cell signaling (Cheng & Nichols, 2016; Lamaze et al., 2017). More recently, a new function of caveolae was established as mechanosensors that play an essential role in cell mechanoprotection both *in vitro* and *in vivo* (Garcia et al., 2017; Y.-W. Lim et al., 2017; Lo et al., 2015; Sinha et al., 2011). The mechanical function of caveolae is likely to explain their particular abundance at the surface of specialized cells that undergo chronic mechanical stress, such as adipocytes, endothelial and muscle cells. Moreover, mutations or abnormal expression of caveolae components have been associated with lipodystrophy, vascular dysfunction, cancer and muscle disorders (reviewed in Lamaze et al., 2017; Le Lay & Kurzchalia, 2005). The molecular mechanisms underlying caveolin-associated diseases remain poorly understood.

In this study, we investigated the mechanical role of caveolae in caveolinopathies, a family of muscle diseases involving mutations in the CAV3 gene. Human caveolinopathies can affect both cardiac and skeletal muscle tissues, and encompass five distinct genetic disorders: Rippling Muscle Disease (RMD), Distal Myopathies (DM), HyperCKemia (HCK), Limb-Girdle Muscular Dystrophy 1C (LGMD-1C), and Familial Hypertrophic Cardiomyopathy

(HCM) (Gazzerro et al., 2010)., These disorders share nevertheless common characteristics such as mild muscle weakness, high levels of serum creatine kinase, variations in muscle fiber size and an increased number of central nuclei as observed in muscle biopsies (Betz et al., 2001; Carbone et al., 2000; Minetti et al., 1998; Tateyama et al., 2002).

We focused our investigations on two representative CAV3 mutations namely Cav3 P28L and Cav3 R26Q, which are respectively responsible for HCK (Merlini et al., 2002), and RMD, HCK and LGMD-1C (Sotgia et al., 2003). These two heterozygous mutations both lead to the abnormal retention of Cav3 in the Golgi complex. The P28L and R26Q Cav3 mutations have so far been associated to deregulations of some signaling pathways (Brauers et al., 2010; Sotgia et al., 2003), defects in membrane repair (Cai et al., 2009; Hernandez-Deviez et al., 2008) and defect in the mechanoprotection of the muscle tissue (Lo et al., 2015). These studies were conducted either *in vivo* in transgenic mice or zebrafish or *in vitro* in the mouse muscle cell line C2C12 or fibroblasts overexpressing a mutant form of Cav3, and the mechanical aspect of caveolae have been poorly explored in this context.

In this study, we investigated whether human myotubes from patients bearing the Cav3 P28L or Cav3 R26Q mutations presented defects in the mechanoresponse mediated by caveolae at the plasma membrane. We also tested the hypothesis that the mechanical flattening of caveolae could be involved in the selective regulation of signaling pathways (Nassey & Lamaze, 2012), by studying interleukin-6 (IL6) signaling, a pathway that plays an essential role in muscle tissue homeostasis (Muñoz-Cánoves et al., 2013).

We show here that the Cav3 P28L and Cav3 R26Q myotubes are unable to assemble *bona fide* caveolae at the plasma membrane, leading to a loss of membrane tension buffering and membrane integrity under mechanical stress. We also found that the absence of functional caveolae impairs the regulation of the IL6/STAT3 pathway in the mutant myotubes both at rest and under mechanical stress. As a result, the IL6 signaling pathway is chronically hyperactivated and several of the STAT3 target genes are highly expressed. Finally, the overexpression of WT Cav3 in mutant myotubes was sufficient to assemble a functional pool of caveolae and rescue mechanosensing and IL6/STAT3 signaling pathway regulation in these cells.

Results

Drastic decreased number of caveolae at the plasma membrane of Cav3 mutant myotubes.

To address the impact of Cav3 mutations in muscle disorders, we analyzed WT, Cav3 P28L and Cav3 R26Q myotubes obtained from immortalized myoblasts which were isolated from healthy or Cav3 mutant patients and differentiated for 4 days. The state of myotube differentiation was validated by the expression level of the differentiation marker MF-20 (myosin II heavy chain) in the three cell lines (Supplementary Fig. 1a). We first analyzed the presence and the ultrastructure of caveolae at the plasma membrane of myotubes by electron microscopy. In WT myotubes, we observed many structures corresponding to *bona fide* caveolae that were directly connected to the plasma membrane or to larger vacuoles of variable size deeper inside the cell known as rosettes, and that could still be connected to the plasma membrane (Fig. 1a). In contrast, few caveolae if any could be detected at the plasma membrane of mutant myotubes, and no large vacuolar structures were observed.

This drastic decrease of caveolae number led us to investigate the localization of Cav3, since it is required for caveolae assembly at the plasma membrane (Minetti et al., 2002). Immunoblot analysis showed a reduced expression of mutant Cav3 as compared to WT (Fig. 1b), with a shifted band for the R26Q mutant corresponding to the Cav3 mutant form, as observed previously (Sotgia et al., 2003). Cav3 immunostaining revealed that WT Cav3 was mainly associated with the plasma membrane of myotubes and also partially localized in the Golgi complex, defined by GM130 staining (Fig. 1c). On the contrary, in the P28L and R26Q myotubes, Cav3 showed a drastic accumulation in the Golgi complex, as shown by the strong colocalization with GM130, in agreement with published data (Brauers et al., 2010; Sotgia et al., 2003). These data confirm that the Cav3 P28L and R26Q mutations retain Cav3 in the Golgi complex, which results in a drastic reduction of the number of caveolae present at the plasma membrane of the Cav3 mutant myotubes.

After 4 days of differentiation, Cav1 could still be expressed in myotubes and could potentially participate to the formation of caveolae independently from Cav3. We therefore analyzed Cav1 expression in myotubes after 4 days of differentiation and found that Cav1 was indeed expressed to the same level in the three cell lines (Supplementary Fig. 1b). We

also found that Cav1 colocalized perfectly with Cav3 at the plasma membrane and to a lesser extent at the Golgi complex in WT myotubes whereas it was mainly retained in the Golgi complex in Cav3 P28L and R26Q myotubes (Supplementary Fig. 1c). This indicates that Cav1 can form hetero-oligomers with Cav3, and that the Cav3 P28L and R26Q mutants are dominant on Cav1 localization.

Cav3 P28L and R26Q myotubes exhibit major defects in membrane tension buffering and mechanoprotection under mechanical stress.

To know whether the strong decrease in caveolae number could induce defects in cell mechanoprotection, we first determined if the Cav3 P28L and R26Q myotubes could buffer the increase of membrane tension induced by a mechanical stress. We thus applied a 45 mOsm hypo-osmotic shock to aligned myotubes and we measured the apparent membrane tension before and after 5 min of hypo-osmotic shock using membrane nanotube pulling with optical tweezers. While the mutant myotubes showed no significant changes in membrane tension in resting condition (Fig. 2a), they showed a significant increase of membrane tension (P28L: $63.1\% \pm 9.8\%$; R26Q: $93.8\% \pm 11.2\%$) under 45 mOsm hypo-osmotic shock compared to WT myotubes ($33\% \pm 9.7\%$) (Fig. 2b). These results clearly show that the Cav3 P28L and R26Q mutant myotubes have lost the ability to buffer membrane tension variations induced by intense mechanical stress.

We next tested whether the lack of membrane tension buffering could result in insufficient mechanoprotection and increased membrane fragility in mechanically challenged mutant myotubes. We designed an assay to quantify the percent of cells that rupture their membrane under mechanical stress. This assay (membrane burst assay) consists in incubating aligned myotubes with calcein-AM, a permeant fluorescent dye (green) that becomes fluorescent only inside the cell, and with the nucleus specific dye DAPI to specifically visualize myotubes (Supplementary Fig. 2b). Live imaging was performed on myotubes subjected for 10 min to 30 mOsm hypo-osmotic shock in the presence of propidium iodide (PI), a non-permeant fluorescent dye (red) that cannot enter cells if the plasma membrane is intact. The concomitant decrease of calcein-AM fluorescence and the appearance of PI fluorescence in the nucleus indicate the loss of membrane integrity (Fig. 2c). After 10 min of hypo-osmotic shock, in comparison to WT myotubes, mutant myotubes not only showed a higher percentage of cells that bursted out (WT: $52.9\% \pm 2.84\%$; P28L: $78.26\% \pm 2.38\%$; R26Q: $88.56\% \pm 1.93\%$) but also a shorter

time of resistance to bursting (WT: 4.5 min \pm 0.2, P28L: 2.1 min \pm 0.1, R26Q: 2.7 min \pm 0.2) (Fig. 2d). Importantly, when we apply a milder hypo-osmotic shock (150 mOsm), for which no increase in membrane tension could be measured, the plasma membrane of all three cell lines remained intact after 10 min of shock (Supplementary Fig. 2b and 2c).

In agreement with the absence of caveolae observed at the plasma membrane of the mutant myotubes (Fig. 1), when WT myotubes were depleted for Cav3, we measured a percentage of bursted out cells that was similar to mutant myotubes (siCtl: 22.7% \pm 1.5%, siCav3: 88.93% \pm 1.4%; Fig. 2e and 2f; Supplementary Fig. 2d). Likewise, Cav3 depleted myotubes significantly bursted out faster as compared to control myotubes (siCtl: 3.1 min \pm 0.2, siCav3: 2.4 min \pm 0.1) (Fig. 2e and 2f). Together, our results demonstrate that the Cav3 P28L and R26Q mutant myotubes, as well as the myotubes depleted for Cav3, are unable to provide the mechanoprotection that is required to maintain the integrity of the plasma membrane under mechanical stress.

Chronic hyperactivation of the IL6/STAT3 signaling pathway in Cav3 P28L and R26Q mutant myotubes.

Considering the key role of caveolae and caveolin in intracellular signaling (Lamaze et al., 2017), we next investigated the possible impact of the loss of functional caveolae on some of the muscle signaling pathways. We focused our analysis on the IL6/STAT3 signaling pathway that has been associated with satellite cells exhaustion and muscle wasting (Bonetto et al., 2011; Price et al., 2014; Tierney et al., 2014). Furthermore, the IL6 signal transducer glycoprotein gp130, which, together with the IL6 receptor subunit, assemble the IL6 receptor, has been localized in caveolae in a myeloma cell line (Podar et al., 2003), suggesting a potential regulation of the IL6 signaling pathway by caveolae. IL6 binding to the IL6 receptor is classically followed by the activation of receptor-bound JAK1 and JAK2 kinases, which in turn phosphorylate the signal transducer and activator of transcription 3 (STAT3) that is then translocated as a dimer to the nucleus where it activates the transcription of IL6 sensitive genes (Heinrich et al., 2003).

We therefore monitored the level of STAT3 tyrosine phosphorylation (pSTAT3) in myotubes stimulated for 5 and 15 min with physiological concentrations of IL6. At steady state, in the absence of IL6 stimulation, little if any phosphorylation of STAT3 was detected in WT myotubes. In contrast, we found that the level of pSTAT3 was already high in Cav3 P28L and R26Q mutant myotubes in the absence of IL6 stimulation. While IL6 stimulation

for 5 and 15 min led to increased levels of pSTAT3 in WT myotubes, we observed a higher level of pSTAT3 in Cav3 P28L and R26Q mutant myotubes at similar times of stimulation (Fig. 3a, b). To rule out a possible contamination of cell lysates by undifferentiated myotubes, we also directly investigated the nuclear translocation of pSTAT3 by immunofluorescence in differentiated myotubes characterized by the presence of multiple nuclei (Fig. 3c, d). Again, we could detect a higher pSTAT3 signal in the nuclei of mutant myotubes as compared to WT at steady state (WT: 1.62 ± 0.1 ; P28L: 2.06 ± 0.2 ; R26Q: 2.02 ± 0.2), and after 15min of IL6 stimulation, although it was less pronounced in P28L mutants (WT: 3.03 ± 0.2 ; P28L: 3.66 ± 0.2 ; R26Q: 4.8 ± 0.4). These data confirm the immunoblot analysis, and show that the IL6/STAT3 signaling pathway is hyperactivated in the Cav3 P28L and R26Q mutant myotubes both at steady state and upon IL6 stimulation.

We next investigated if the regulation of the IL6/STAT3 pathway was dependent on the presence of functional caveolae at the plasma membrane and thus on the expression of Cav3. We therefore repeated the experiments on WT myotubes depleted for Cav3. By immunoblot analysis, we found that Cav3 depletion by siRNA led also to an hyperactivation of the IL6 pathway with an overall activation of STAT3 (Fig. 3e, f) (0 min: siCtl: 0.11 ± 0.1 , siCav3: 0.2 ± 0.1 ; 5 min: siCtl: 0.33 ± 0.2 , siCav3: 0.98 ± 0.4 ; 15 min: siCtl: 0.4 ± 0.1 , siCav3: 2.12 ± 0.2). These results indicate that Cav3 is a negative regulator the IL6/STAT3 pathway in myotubes and that depletion of Cav3 in WT myotubes reproduces the phenotype of the Cav3 mutant phenotypes. It demonstrates that the absence of Cav3 and/or caveolae at the plasma membrane of mutant myotubes is responsible for the constitutive hyperactivation of the IL6/STAT3 signaling pathway.

STAT3 is a key transcription factor which controls the transcription of many downstream genes whose products mediate the pleiotropic effects of STAT3 in physiological and pathological contexts (Levy & Lee, 2002). We therefore examined the consequences of IL6/STAT3 chronic activation on gene expression. In the context of muscle diseases, we investigated the transcription of muscle-specific genes as STAT3 was suggested to be involved in their regulation of (Bonetto et al., 2011). We focused our analysis on *FBXO32* a gene known to be associated with muscle atrophy, and on the *ACTC1*, *MYH8* and *SOCS3* genes that are associated with muscle regeneration. *SOCS3* serves also as a positive control, as it is transcribed upon STAT3 activation and its gene product *SOCS3* is a major actor in the negative regulation of the pathway (Heinrich et al., 2003). Using quantitative PCR, we found an increased transcription of *SOCS3*, *MYH8* and

ACTC1 genes, and little effect on the *FBXO32* gene (*SOCS3*: WT 1, P28L 2.5 ± 0.58 , R26Q: 2.95 ± 1.1 ; *FBXO32*: WT 1, P28L 2.33 ± 1.01 , R26Q: 1.65 ± 0.59 ; *MYH8*: WT 1, P28L $15,15 \pm 4,3$, R26Q $5,24 \pm 1,6$; *ACTC1*: WT 1, P28L $3,37 \pm 1,18$, R26Q $12,3 \pm 4,56$) (Fig. 3g). These data strongly suggest that the high level of pSTAT3 found in the Cav3 P28L and R26Q mutant myotubes is responsible of the deregulation of several genes that could be involved in the muscular pathology.

IL6/STAT3 mechanosignaling is impaired in Cav3 P28L and R26Q mutant myotubes.

If caveolae and caveolins have often been associated with signalin (Jade P.X. Cheng & Nichols, 2016; Lamaze et al., 2017), the integration of this function with their role in mechanical stress has not been explored. We have proposed that the mechano-dependent cycle of caveolae disassembly and reassembly could be linked to the regulation of signaling by caveolae (Lamaze et al., 2017; Nassoy & Lamaze, 2012). We thus analyzed whether the regulation of the IL6/STAT3 pathway by caveolae could depend on mechanical stress. When myotubes were subjected to hypo-osmotic shock and stimulated with IL6, we observed a dramatic decrease of STAT3 activation (approx. 80%) in WT myotubes whereas no significant changes were observed in Cav3 P28L and R26Q mutant myotubes (Fig. 4a, b). We also tested the effect of mechanical stretching on IL6/STAT3 signaling as this is more relevant to the kind of stress experienced by skeletal muscles during exercise. When we applied a 10% cyclic stretch for 30 min to WT myotubes followed by IL6 stimulation, we also observed a drastic reduction of STAT3 activation, confirming that the IL6/STAT3 pathway is tightly regulated by mechanical cues (Supplementary Fig. 3). To know whether the mechanical regulation of IL6 signaling requires the presence of functional caveolae, we also applied an hypo-osmotic shock to WT myotubes depleted of Cav3. While no effect was observed at steady state, we found that STAT3 activation was slightly decreased by mechanical stress (approx. 20%) in WT myotubes when no changes were observed in Cav3 depleted myotubes (Fig. 4c-d). The poor adhesion of Cav3 P28L and R26Q mutant myotubes on the stretching membrane did not allow to perform cycling stretching. Nevertheless, these results confirm that the IL6 pathway is negatively regulated by mechanical stress in myotubes and that this regulation is lost in the absence of functional caveolae as shown in Cav3 P28L and R26Q mutant myotubes and in WT myotubes depleted for Cav3. It further confirms that the defects of mechanoprotection and IL6/STAT3

signaling regulation observed in the Cav3 P28L and R26Q mutant myotubes result from the lack of caveolae assembly at their plasma membrane.

Re-expression of WT Cav3 rescues a normal phenotype in Cav3 P28L myotubes.

Our experiments show that the depletion of Cav3 in WT myotubes reproduces the defects observed in P28L and R26Q myotubes, implying that the absence of Cav3 at the plasma membrane, as a result of its abnormal retention in the Golgi complex, is responsible for the observed phenotype. To validate this hypothesis, we generated stable WT and P28L myoblasts expressing either GFP or Cav3 WT tagged with GFP (Cav3-GFP). Immunofluorescent microscopy confirmed that re-expressed Cav3-GFP was mainly localized at the plasma membrane whereas endogenous Cav3 remained colocalized with the Golgi marker GM130 in Cav3-GFP P28L myotubes (Fig. 5a and supplementary Fig. 4a). We next performed electron microscopy to see if Cav3 WT re-expression would allow to reconstitute a pool of structurally defined caveolae at the plasma membrane of Cav3 P28L myotubes expressing GFP or Cav3 GFP. While the plasma membrane of control GFP myotubes presented very few, often isolated, caveolae structures, Cav3 rescued myotubes presented higher amount of *bona fide* caveolae, including larger vacuolar structures with connected caveolae i.e. rosettes, as observed above in WT myotubes (Fig. 5b and 1c). These observations confirm that the decrease of caveolae numbers in Cav3 P28L myotubes is a direct consequence of the retention of Cav3 P28L in the Golgi complex.

The next question was to know whether the reconstitution of the caveolae reservoir at the plasma membrane of Cav3 P28L myotubes was sufficient to rescue the mechanoprotection and mechanosignaling properties. We therefore monitored the resistance to bursting of P28L GFP and P28L Cav3-GFP myotubes as described above. Notably, Cav3-GFP P28L myotubes showed a dramatic increase in the resistance to bursting under hypo-osmotic shock as compared to P28L GFP myotubes (P28L GFP: $49.01\% \pm 2.7\%$; P28L Cav3-GFP: $18.44\% \pm 2.1\%$). It also took a significantly longer time for P28L Cav3-GFP myotubes to burst out as compared to P28L GFP myotubes (GFP: $1.63 \text{ min} \pm 0.09$; Cav3-GFP: $2.28 \text{ min} \pm 0.21$) (Fig. 5c, d). Finally, we analyzed the regulation of the IL6/STAT3 pathway by monitoring STAT3 phosphorylation and nuclear translocation in P28L GFP and P28L Cav3-GFP myotubes. At steady state, we observed a significant decrease of pSTAT3 activation and nuclear translocation in Cav3-GFP myotubes as compared to GFP myotubes, indicating that the re-expression of Cav3 was sufficient to

reduce the hyperactivation of STAT3 observed at steady state in Cav3 P28L myotubes (P28L GFP: 2.53 ± 0.2 , Cav3-GFP: 1.7 ± 0.1) (Fig 5e, f). Interestingly, we also found that the re-expression of Cav3-GFP in WT myotubes led to a decrease of pSTAT3 activation and nuclear translocation at steady state as compared to non transfected WT myotubes (Supplementary Fig. 4c). Altogether, these results show that Cav3 expression and localization at the plasma membrane of P28L myotubes was sufficient to rescue the lack of membrane integrity and the overactivation of the IL6 pathway described before. This allows us to conclude that the phenotype observed in mutant myotubes is due to a lack of Cav3 and caveolae at the plasma membrane.

Discussion

The first Cav3 mutations associated with muscle disorders were described 20 years ago (Minetti et al., 1998). Since then, a lot of effort has been put in trying to understand how these mutations could alter muscle tissue integrity and cause the observed syndromes. Due to its localization at the plasma membrane of myotubes and at the sarcolemma of muscle fibers, Cav3 has been showed to regulate myoblast fusion (Bjerregard et al., 2014) and T-tubule organization (Ferruccio Galbiati et al., 2001). Also, Cav3 interacts with the dystrophin complex (Song et al., 1996) and regulates the trafficking of dysferlin (Hernández-Deviez et al., 2006.), two proteins causing severe myopathies when deregulated. Furthermore, Cav3 is involved in the regulation of different signaling pathways important for muscle function such as calcium (Weiss et al., 2008), the insulin/GLUT4/Akt pathway (Fecchi et al., 2006) or TrkA and EGFR signaling (Brauers et al., 2010). Finally, our group showed a role of Cav3 in membrane tension buffering and mechanoprotection of human myotubes (Sinha et al., 2011). Furthermore, a recent study showed in an *in vivo* model of zebrafish expressing the R26Q mutation, that Cav3 was required for membrane integrity of muscle tissue (Lo et al., 2015).

In the present work, we studied human myotubes after differentiation from myoblasts that were isolated and immortalized from healthy patients or patients bearing either the P28L or the R26Q mutation. As it is likely that Cav3 and caveolae regulate several functions in myotubes, we chose to focus on two major but poorly explored functions of caveolae that could have an important impact on the integrity of muscle cells and tissue. We first studied mechanoprotection as this function recently described has not been extensively investigated in muscle cells and never in human myotubes. We demonstrate in this study that the Cav3

P28L and R26Q myotubes show a strong defect in mechanoprotection, with less resistance of the plasma membrane to mechanical stress. We show that this defect results from the absence of a caveolae reservoir at the plasma membrane which leads to a lack of membrane tension buffering under mechanical stress.

We then studied the regulation of the IL6/STAT3 signaling pathway in resting myotubes or under mechanical stress. Indeed, we have proposed that the cycles of caveolae disassembly and reassembly induced by mechanical stress could have an impact on some signaling pathways (Nassoy & Lamaze, 2012). We chose to investigate the IL6 pathway as it is tightly associated to mechanical stress in muscle cells, and IL6 is mostly secreted during exercise (Kenneth Ostrowski et al., 1998). We could show for the first time that the Cav3 mutant myotubes present a deregulation of the IL6/STAT3 signaling pathway, as this pathway hyperactivated in these myotubes under resting conditions. This defect translated into increased gene expression of *MYH8*, *SOCS3* and *ACTC1*, genes that have been associated with muscle regeneration, a process that may be important for the regulation of muscle tissue size. Whether the regulation of these genes depends directly on STAT3 still needs to be demonstrated. In addition, we show that the IL6 signaling pathway is negatively regulated by mechanical stress in a Cav3 dependent manner, and that this mechano-regulation is lost in mutated myotubes. It is likely that this mechano-regulation of the IL6/STAT3 pathway allows a negative feedback loop to avoid a chronic activation of the pathway and to adapt it to the mechanical stress that myotubes constantly experience during their lifetime.

Importantly, we also found that Cav3 mutant myotubes phenocopy Cav3 depleted myotubes. Furthermore, the defects in mechanoprotection and IL6 signaling regulation could be, at least partially, rescued in Cav3 P28L myotubes re-expressing the WT form of Cav3 and thereby reassembling functional caveolae at the plasma membrane. In conclusion, our data also confirm that the retention of the Cav3 mutated forms in the Golgi complex is responsible for the absence of functional and morphologically defined caveolae at the plasma membrane of mutated myotubes. This absence leads both to deficient mechanoprotection and impaired mechano-regulation of the IL6/STAT3 signaling pathway, a pathway tightly linked to muscle mass and size. Mechanoprotection and proper regulation of muscle size are two features extremely important for general muscle integrity and it is easy to understand how alteration of these two processes can be deleterious for muscle tissue integrity, as it is often seen in muscular dystrophic patients.

Methods

Cell lines. P28L and R26Q human myoblasts were immortalized by the platform for immortalization of human cells of the Institute of Myology as described in (Mamchaoui et al., 2011). Briefly, myoblasts were transduced with lentiviral vectors encoding hTERT and cdk4 and containing puromycin (P28L) or puromycin and neomycin (R26Q) selection markers. Transduced cells were selected with puromycin (1 µg/ml) for 6 days (P28L) or with puromycin (1 µg/ml) for 6 days and neomycin (1 mg/ml) for 10 days (R26Q). Cells were seeded at clonal density, and individual myogenic clones were isolated.

For caveolin-3 expression, immortalized WT and P28L myoblasts were transduced with lentiviral vectors expressing WT caveolin-3 and a GFP reporter gene (MOI 5). A GFP lentiviral vector was used as control (MOI 5).

Cell culture. All cells were grown at 37°C under 5% of CO₂. All myoblasts cell lines were cultured in Skeletal Muscle Cell Growth Medium (Promocell) supplemented with 20% FCS (Gibco, Life technologies), 50 µg/mL of fetuine, 10 ng/mL of epidermal growth factor, 1 ng/mL basic fibroblast growth factor, 10 µg/mL of insulin and 0,4 µ/mL of dexamethasone (Promocell). Prior to any cell seeding, surfaces (well, coverslip, patterned coverslips) are coated with 0.01% of matrigel (v/v) (Sigma) for 15 min at 37°C. For myoblast differentiation, confluent cells (80-100% confluency) are put in DMEM high-glucose Glutamax (Gibco, Life Technologies), supplemented with 0.1% of insulin (v/v) (Sigma) for 4 days.

Antibodies and reagents. Mouse anti-αTubulin (Sigma-Aldrich, clone B512, T5168, 1/1000 for WB); mouse anti-caveolin-3 (Santa cruz, clone A3, sc-5310, 1/1000 for WB, 1/250 for IF); rabbit anti-caveolin-1 (Cell signaling, 3238, 1/1000 for WB, 1/500 for IF); goat anti-GM130 (Santa Cruz, clone P-20, sc-16268, 1/50 for IF); mouse anti-MF20 (kind gift of Vincent Mouly, 1/100 for WB, 1/20 for IF); mouse anti-STAT3 (Cell signaling, clone 124H6, 9139, 1/1000 for WB); rabbit anti-pSTAT3 (Cell signaling, clone D3A7, 9145, 1/1000 for WB, 1/75 for IF); Secondary antibodies conjugated to Alexa FITC, Cy3, Cy5 or horseradish peroxidase (Beckman Coulter or Invitrogen). DAPI (Sigma-Aldrich).

RNA interference-mediated silencing. Myoblasts were transfected with small interfering RNAs (siRNAs) using HiPerFect (Qiagen) according to the manufacturer's instructions at

days 0 and 2 of differentiation and were cultured in differentiation medium for a total of 4 days. Experiments were performed on validation of silencing efficiency by immunoblot analysis using specific antibodies and normalizing to the total level of tubulin used as loading controls. 20 nM of a pool of four siRNA targeting Cav3 were used (SI03068730, SI02625665, SI02625658 and SI00146188, QIAGEN), Control siRNA (1022076, QIAGEN) was used at the same concentration and served as reference point.

Immunoblotting. Cells were lysed in sample buffer (62.5 mM Tris/HCl pH 6.0, 2% v/v SDS, 10% glycerol v/v, 40mM dithiothreitol and 0.03% w/v phenol red). Lysates were analyzed by SDS-PAGE and Western blot analysis and immunoblotted with the indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies. Chemiluminescence signal was revealed using Pierce™ ECL Western Blotting Substrate, SuperSignal West Dura Extended Duration Substrate or SuperSignal West Femto Substrate (Thermo Scientific Life Technologies). Acquisition and quantification were performed with the ChemiDoc MP Imaging System (Bio-rad).

Immunofluorescence. Myoblasts were grown and differentiated on coverslips for 4 days. For Cav3, Cav1, MF-20, GM130 staining, cells are fixed with 4% PFA (v/v) (Sigma-Aldrich) for 10min at RT, quenched in 50 mM NH₄Cl and then permeabilized with 0.2% BSA (v/v) and 0.05% saponin (v/v) (Sigma-Aldrich) in PBS for 20 min. Cells are incubated sequentially with indicated primary and fluorescence-conjugated secondary antibody in permeabilization buffer for 1h at RT. For pSTAT3 staining, cells are fixed and permeabilized with cold methanol for 15 min at -20°C. After washes with PBS 0.2% BSA (v/v), cells are incubated sequentially with indicated primary and fluorescence-conjugated secondary antibody in PBS 0.2% (v/v) for 1h at RT. In both protocols, coverslips are mounted in Fluoromount-G mounting medium (eBioscience) supplemented with 2 µg/mL of DAPI (Sigma-Aldrich). Acquisition of images are done using a spinning disk microscope (inverted Spinning Disk Confocal Roper/Nikon; Camera: CCD 1392x1040 CoolSnap HQ2 ; objective : 60x CFI Plan Apo VC).

Electron microscopy. Epon embedding was used to preserve the integrity of cell structures. Myotubes were fixed sequentially for 1 hour at room temperature with 1.25% glutaraldehyde in 0.1 M Na-Cacodylate and then overnight at 4°C.

Cells were washed extensively with 0.1 M Na-Cacodylate pH 7.2. Membrane fixation was performed for 1 h at room temperature with 1% OsO₄ in 0.1 M Na-Cacodylate pH 7.2. Cells were dehydrated by incubation with aqueous solutions of ethanol at increasing concentrations (50, 70, 90, then 100%, each for 10 min at room temperature). Embedding was finally performed in LX112 resin. Cells were infiltrated with a 1:1 LX112:ethanol solution, washed with LX112, and embedded overnight at 60°C in LX112 resin. Ultrathin 65 nm sections were sliced using a Leica UCT ultramicrotome and mounted on nickel formvar/carbon-coated grids for observations. Contrast was obtained by incubation of the sections for 10 min in 4% uranyl acetate followed by 1 min in lead citrate.

Electron micrographs were acquired on a Tecnai Spirit electron microscope (FEI, Eindhoven, The Netherlands) equipped with a 4k CCD camera (EMSIS GmbH, Münster, Germany)

Micropatterning. 18 mm coverslips were micropatterned as described in (Carpi et al., 2011) using a photo-mask with lines of 10 µm of width, separated by 60 µm. In both force measurements and membrane bursting assay, myoblasts are plated at confluency on line micropatterns coverslips coated with 0.01% of matrigel (v/v) (Sigma) for 15 min at 37°C. Differentiation of myoblasts is achieved as described above in section *Cell culture*.

Force Measurements. Plasma membrane tethers were extracted from cells by a concanavalin A (Sigma-Aldrich) coated bead (3 µm in diameter, Polysciences) trapped in optical tweezers. The optical tweezers are made of a 1064 nm laser beam (ytterbium fiber laser, $\lambda = 1064$ nm, TEM 00, 5 W, IPG Photonics, Oxford, MA) expanded and steered (optics by Elliot Scientific, Harpenden, UK) in the back focal plane of the microscope objective (Apo-TIRF 100× NA 1.45, Nikon). The whole setup was mounted on a Nikon Eclipse-Ti inverted microscope. The sample was illuminated by transmitted light, and movies were acquired at 10 Hz with an EM-charge-coupled device camera (Andor iXon 897) driven by Micro-Manager. The fine movements and particularly the translational movement necessary to pull the membrane tether were performed using a custom-made stage mounted on a piezoelectric element (P753, Physik Instrumente, Karlsruhe, Germany) driven by a servo controller (E665, Physik Instrumente) and a function generator (Sony Tektronix AFG320).

Calibration was performed using an oscillatory modulation driven by a function generator and measuring the response of the bead to an oscillatory motion of the stage. We measured $k = 16P \text{ pN}/(\mu\text{m}\cdot\text{W})$, where P is the laser power. This relationship is linear in the laser power range used for the experiments (0.4–2 W).

The membrane tether was held at constant length to measure the static force. For measuring membrane tension changes due to hypo-osmotic shock, a first tether was first pulled at 300 mOsm (iso condition). A second tube was pulled on the same cell 5 minutes after diluting the medium with milliQ water to obtain 45 mOsm. The position of the bead used to compute tether forces was detected from the images using a custom ImageJ macro.

Membrane burst assay. Line micropatterned myotubes are incubated in 5 $\mu\text{g}/\text{mL}$ of calcein-AM (Life technologies) and 50 $\mu\text{g}/\text{mL}$ of DAPI (Sigma-Aldrich) for 15 min at 37°C in the dark. Medium was then switched back with differentiation medium to wash out the excess of calcein-AM. The medium is then switched again with a 30 mOsm hypo-osmotic shock medium obtained after a dilution of 10% medium and 90% H₂O, supplemented with 2 mg/mL of PI (Sigma). Immediately after medium switching, pictures are taken every minute for 10 min using a videomicroscope (Inverted microscope Nikon Ti-E, Camera: CCD 1392x1040 CoolSnap HQ2, objective: 10x CFI Fluor).

IL6 stimulation. Myotubes were starved 4h by switching the differentiation medium to DMEM medium. In resting conditions, cells are then stimulated by switching the medium with DMEM with 0.2% BSA (w/v), supplemented with 10ng/mL of human recombinant IL6 (R&D) for 0, 5 or 15min at 37°C. For hypo-osmotic conditions, medium was first switched to 75% hypo-osmotic shock (25% DMEM, 75% H₂O) for 5min and then switched to the same medium supplemented with 10ng/mL of IL6 for 5 more minutes at 37°C. For stretching conditions, myoblasts were differentiated on fibronectin (Sigma-Aldrich) coated stretchable plates (Uniflex® culture plate, Flexcell International) and were then subjected or not to 30min of cyclic stretch (10% elongation, 0.5Hz), using the FX-4000T TM Tension Plus device (Flexcell International), followed by 0min or 5min of IL6 stimulation. Cells are lysed and samples are analyzed by immunoblotting. For the analysis, pSTAT3 levels were quantified by calculating the ratio between pSTAT3 and STAT3, both normalized to Tubulin signal. For the analysis of pSTAT3 nuclear translocation, myotubes were differentiated on coverslips, stimulated with IL6 as described above for 0min or 15min and were then fixed for

further immunofluorescence analysis. Quantification corresponds to the ratio between the mean pSTAT3 intensity in the nuclei on the one in the cytoplasm.

Quantitative PCR. Cells were lysed and RNA extraction was performed using an extraction kit (RNeasy Plus, Qiagen). Reverse-transcription reaction was performed with 1µg of RNA per reaction, using high capacity cDNA reverse-transcription kit (Applied biosystem). qPCR was performed on 50ng of cDNA for a reaction in a total volume of 20µL, using Taqman Gene Expression Assays (GAPDH: Hs02786624_g1 ; FBXO32: Hs01041408_m1 ; ACTC1: Hs01109515_m1 ; MYH8: Hs00267293_m1 ; SOCS3: Hs02330328_s1, Applied biosystem) and a Lightcycler 480 Probes Master kit (Roche). Relative expression levels were calculated using $\Delta\Delta CT$ method with fold changes calculated as $2^{-\Delta\Delta CT}$

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Figure legends

Figure 1 | Characterization of caveolae and Cav3 expression in WT and Cav3 P28L and Cav3 R26Q myotubes. (a) Electron micrographs of WT, Cav3 P28L or Cav3 R26Q myotubes. Caveolae and interconnected caveolae are indicated with arrowheads and asterisks, respectively. (b) Immunoblot analysis of total levels of Cav3 in WT, Cav3 P28L and Cav3 R26Q differentiated myotubes. Tubulin serves as loading control. (c) Immunofluorescent labeling of Cav3 and GM130 in WT, Cav3 P28L or Cav3 R26Q myotubes analyzed by confocal microscopy. Arrows in inset indicate the plasma membrane and arrowheads indicate the Golgi complex. (a) Scale bar = 200 nm. (c) Scale bar = 10 μ m. Reproducibility of experiments: (a) Representative cells. (b) and (c) Representative data for 3 experiments.

Figure 2 | Cav3 P28L and Cav3 R26Q myotubes present defects in membrane tension buffering and membrane integrity. (a, b) Membrane tension measurements analysis using optical tweezers and nanotube pulling on aligned WT, Cav3 P28L or Cav3 R26Q myotubes. Membrane tethers were pulled in the perpendicular axis of myotubes after micropatterning in resting conditions and 5 min after a 45 mOsm hypo-osmotic shock (a, b, left panels). Membrane tension was analyzed in resting condition (a, right panel) and the difference of membrane tension before and after hypo-osmotic shock was calculated, reflecting the percentage of increase of membrane tension upon mechanical stress (b, right panel).

(c, e) Micropatterned WT, Cav3 P28L or Cav3 R26Q myotubes (c) WT ctl (siCtl) and Cav3-depleted (siCav3) myotubes (e) were loaded with calcein-AM (green). The medium was switched with a 30 mOsm medium supplemented with propidium iodide (PI, red). Representative pictures were taken at the indicated times during hypo-osmotic shock. Arrows correspond to myotubes and asterisks correspond to bursted myotubes. (d, f) Quantification of the percentage of bursted myotubes (upper panel) and mean time of bursting in minutes (lower panel) in (c) and (e), respectively. (a, b) Scale bar = 5 μ m, (c, e). Scale bar = 120 μ m. Reproducibility of experiments: (a) Representative pictures and quantifications from 7 independent experiments (WT n=20 Cav3 P28L n=23 and R26Q n=22) (b) Representative pictures and quantifications from 7 independent experiments (WT n=17 P28L n=20 and R26Q n=18). (c) Representative data of 3 independent experiments

quantified in **(d)** (% bursted cells: WT n=310, P28L n=299 and R26Q n=271; mean time of bursting: WT n=165, P28L n=233 and R26Q n=240). **(e)** Representative data of 3 independent experiments quantified in **(f)** (% bursted cells: siCtl n=749 and siCav3 n=569 ; mean time of bursting: siCtl n=171 and siCav3 n=506). Mean value \pm SD. **(a, b)** Statistical analysis were done using Kurskal-Wallis test. **(d, f)** Statistical analysis with two-tailed unpaired t test *** P<0,0001

Figure 3 | Hyperactivation of the IL6/STAT3 signaling pathway in Cav3 P28L and Cav3 R26Q. **(a)** Immunoblot analysis of pSTAT3 and STAT3 levels in WT, Cav3 P28L or Cav3 R26Q myotubes stimulated for the indicated times with 10 ng/mL IL6. Tubulin serves as loading control. **(b)** Quantification of STAT3 activation of **(a)**, corresponding to the ratio pSTAT3 on STAT3 total levels after normalization to tubulin levels. **(c)** Confocal microscopy of immunofluorescent pSTAT3 in WT, Cav3 P28L or Cav3 R26Q myotubes stimulated or not for 15 min with 10 ng/mL IL6. **(d)** Quantification of pSTAT3 nuclear translocation in **(c)** corresponding to nuclei/cytoplasm mean intensity ratio of pSTAT3. **(e)** Immunoblot analysis of pSTAT3 levels in WT ctl (siCtl) and Cav3-depleted (siCav3) myotubes stimulated for the indicated times with 10 ng/mL IL6. **(f)** Quantification of STAT3 activation in **(e)**, corresponding to the ratio pSTAT3 on STAT3 total levels after normalization to tubulin levels. **(g)** Expression of STAT3 related genes: from left to right *FBXO32*, *SOCS3*, *MYH8* and *ACTC1* in WT, Cav3 P28L or Cav3 R26Q myotubes. **(c)** Scale bar = 10 μ m. Reproducibility of experiments: **(a, c and e)** Representative data. **(b)** Quantification was done on 4 independent experiments. **(d)** Quantification was done on 3 independent experiments (0 min: WT n=41 P28L n=25 R26Q n=21; 15 min: WT n=22 P28L n=30 R26Q n=30). **(f)** Quantification was done on 4 experiments. **(g)** Quantification was done on 6 (*FBXO32*), 5 (*SOCS3*), 8 (*MYH8*) and 3 (*ACTC1*) independent experiments. Mean value \pm SEM. **(b, f)** Statistical analysis with two-tailed paired t test, **(d, g)** Statistical analysis with two-tailed unpaired t test * P<0,005 ** P<0,001*** P<0,0001, ns non significant

Figure 4 | IL6/STAT3 mechanosignaling is impaired in Cav3 P28L and R26Q myotubes. **(a, c)** Immunoblot analysis of pSTAT3 and STAT3 levels in WT, Cav3 P28L or Cav3 R26Q myotubes **(a)** WT ctl (siCtl) or Cav3-depleted (siCav3) myotubes **(c)** subjected or not to a 75 mOsm hypo-osmotic shock for 10 min, followed by stimulation or not with 10 ng/mL IL6 for 5 min. Tubulin serves as loading control. **(b, d)** Quantification of STAT3

activation in (a) and (c) respectively, corresponding to the ratio pSTAT3 on STAT3 total levels after normalization to tubulin levels.

Reproducibility of experiments: (a, b) Representative data. (b) Quantification was done on 5 and 3 independent experiments for WT, mutants respectively. (d) Quantification was done on 4 independent experiments. Mean value \pm SEM. (b, d) Statistical analysis with two-tailed paired t test * $P < 0,005$, ns non significant

Figure 5 | Overexpression of WT Cav3 rescues a normal phenotype in Cav3 P28L and R26Q myotubes.

(a) Immunofluorescent labeling of Cav3 and Golgi marker GM130 in Cav3 P28L GFP or P28L Cav3-GFP transduced myotubes analyzed by confocal microscopy. Arrows in inset indicate the plasma membrane and arrowheads indicate the Golgi complex. (b) Electron micrographs of Cav3 P28L GFP or P28L Cav3-GFP transduced myotubes. Caveolae and interconnected caveolae are indicated with arrowheads and asterisks, respectively. (c) Micropatterned Cav3 P28L GFP or P28L Cav3-GFP transduced myotubes were loaded with calcein-AM (green). The medium was switched with a 30 mOsm medium supplemented with propidium iodide (PI, red). Representative pictures were taken at the indicated times during hypo-osmotic shock. Arrows correspond to myotubes and asterisks correspond to bursted myotubes. (d) Quantification of the percentage of bursted myotubes (upper panel) and mean time of bursting in minutes (lower panel) in (c). (e) Confocal microscopy of immunofluorescent pSTAT3 in Cav3 P28L GFP or P28L Cav3-GFP transduced myotubes stimulated or not for 15 min with 10 ng/mL IL6. (f) Quantification of pSTAT3 nuclear translocation in (e) corresponding to nuclei/cytoplasm mean intensity ratio of pSTAT3. (a) Scale bar = 10 μ m. (b) Scale bar = 200 nm. (c) Scale bar = 120 μ m. (e) Scale bar = 10 μ m. Reproducibility of experiments: (a) Representative pictures of 3 experiments. (b) Representative pictures (c) Show representative data of 3 experiments quantified in (d) (% burst cells: GFP n=353 and Cav3-GFP n=358; time of burst: GFP n=175 and cav3-GFP n=65). (e) Show representative data of 3 experiments quantified in (f) (0min: GFP n=33 and Cav3-GFP n=42; 15min: GFP n=14 and Cav3-GFP n=13). Mean value \pm SEM. (d) and (f) Statistical analysis with a two-tailed unpaired t test ** $P < 0,01$, *** $P < 0,0001$, ns= non significant

Supplementary figure 1 | MF20 and Cav1 expression in WT, Cav3 P28L and R26Q myotubes. (a, b) Immunoblot analysis of total levels of MF20 (a) and Cav1 (b) in WT, Cav3 P28L or Cav3 R26Q myotubes. Tubulin serves as loading control. (c) Cav3, Cav1 and Golgi marker GM130 immunofluorescence were analyzed by confocal microscopy in WT, Cav3 P28L or Cav3 R26Q myotubes. (a), (b) and (c) Representative data for 3 experiments. (c) Scale bar = 10 μ m

Supplementary figure 2 | Efficient membrane tension buffering and meachanoprotection in Cav3-P28L and Cav3-R26Q myotubes under mild hypo-osmotic shock. (a) Calcein-AM and DAPI fluorescence of WT, Cav3 P28L or Cav3 R26Q myotubes prior to hypo-osmotic shock in the membrane burst assay described in **Figure 2c**. Insets show DAPI in myotubes indicated with arrows in **Figure 2c**. (b) Micropatterned WT, Cav3 P28L or Cav3 R26Q myotubes were loaded with calcein-AM (green). The medium was switched with a 150 mOsm medium supplemented with propidium iodide (PI, red). Representative pictures were taken at the indicated times during hypo-osmotic shock. Arrows correspond to myotubes and asterisks correspond to bursted myotubes. (c) Membrane tension measurements analysis using optical tweezers and nanotube pulling on aligned WT, Cav3 P28L or Cav3 R26Q myotubes. Membrane tethers were pulled in the perpendicular axis of myotubes after micropatterning in resting conditions and 5 min after a 150 mOsm hypo-osmotic shock (upper pane). Membrane tension was analyzed in resting condition (lower panel, left) and the difference of membrane tension before and after hypo-osmotic shock was calculated, reflecting the percentage of increase of membrane tension upon mechanical stress (lower panel, right) (d) Immunoblot analysis of Cav3 depletion in **Figure 2e**. (a,b) Scale bar = 120 μ m. (c) Scale bar = 5 μ m Reproducibility of experiments: (c) Quantifications were done on 5 independents experiments (WT n=17, P28L n=16, R26Q n=14). Mean value \pm SD. Statistical analysis were done using Kruskal-Wallis test. ns non significant

Supplementary figure 3 | IL6/STAT3 signaling in WT myotubes under cyclic stretch. (a) Immunoblot analysis of pSTAT3 and STAT3 levels in WT myotubes subjected or not to 30 min cyclic stretch. Myotubes were then stimulated or not with 10 ng/mL IL6 for 5 min. Tubulin serves as loading control. (b) Quantification of STAT3 activation in (a) corresponding to the ratio pSTAT3 on STAT3 total levels after normalization to tubulin

levels. Reproducibility of experiments: **(b)** Quantifications were done on 3 experiments. Mean value \pm SEM. Statistical analysis were done using two-tailed paired t test * $P < 0,05$

Supplementary figure 4 | Effect of Cav3 overexpression in mechanoprotection and IL6 signaling in WT myotubes. **(a)** Immunofluorescent labeling of Cav3 and Golgi marker GM130 in WT GFP or WT Cav3-GFP transduced myotubes analyzed by confocal microscopy. Arrows in inset indicate the plasma membrane and arrowheads indicate the Golgi complex. **(b)** Quantification of the percentage of bursted myotubes after a 30 mOsm hypo-osmotic shock (left panel) and mean time of bursting in minutes (right panel) in **(a)**. **(c)** Quantification of pSTAT3 nuclear translocation in WT GFP or WT Cav3-GFP transduced myotubes stimulated or not for 15 min with 10 ng/mL IL6, corresponding to nuclei/cytoplasm mean intensity ratio of pSTAT3. **(a)** Scale bar = 10 μ m. Reproducibility of experiments: **(a)** Representative data from 3 independent experiments. **(b)** Quantification done on 3 independent experiments (% burst: GFP n=714, Cav3-GFP n=610; time of burst: GFP n=80, Cav3-GFP n=171). **(c)** Quantification was done on 3 independent experiments (0min: GFP n=16, Cav3-GFP n=21; 15min: GFP n=16, Cav3-GFP n=21). **(b, c)** Statistical analysis were done using two-tailed unpaired t test *** $P < 0,0001$ * $P < 0,05$ ns non significant

Contribution of the authors who performed the experiments

All cell lines were generated and provided by Anne Bigot

Figure 1:

- a) Co-performed by Valérie Chambon (sample preparation and EM) and Melissa Dewulf (cell and sample preparation)
- b, c) Performed by Melissa Dewulf

Figure 2:

- a, b) Co-Performed by Christine Viaris (nanotube pulling) and Melissa Dewulf (micropatterning and cell preparation)
- c-f) Performed by Melissa Dewulf

Figure 3:

Performed by Melissa Dewulf

Figure 4:

Performed by Melissa Dewulf

Figure 5:

- a, c-f) Performed by Melissa Dewulf
- b) Co-performed by Valérie Chambon (sample preparation and EM) and Melissa Dewulf (cell and sample preparation)

Supplementary figure 1:

Performed by Melissa Dewulf

Supplementary figure 2:

- a, b, d) Performed by Melissa Dewulf
- c) Co-Performed by Christine Viaris (nanotube pulling) and Melissa Dewulf (micropatterning and cell preparation)

Supplementary figure 3:

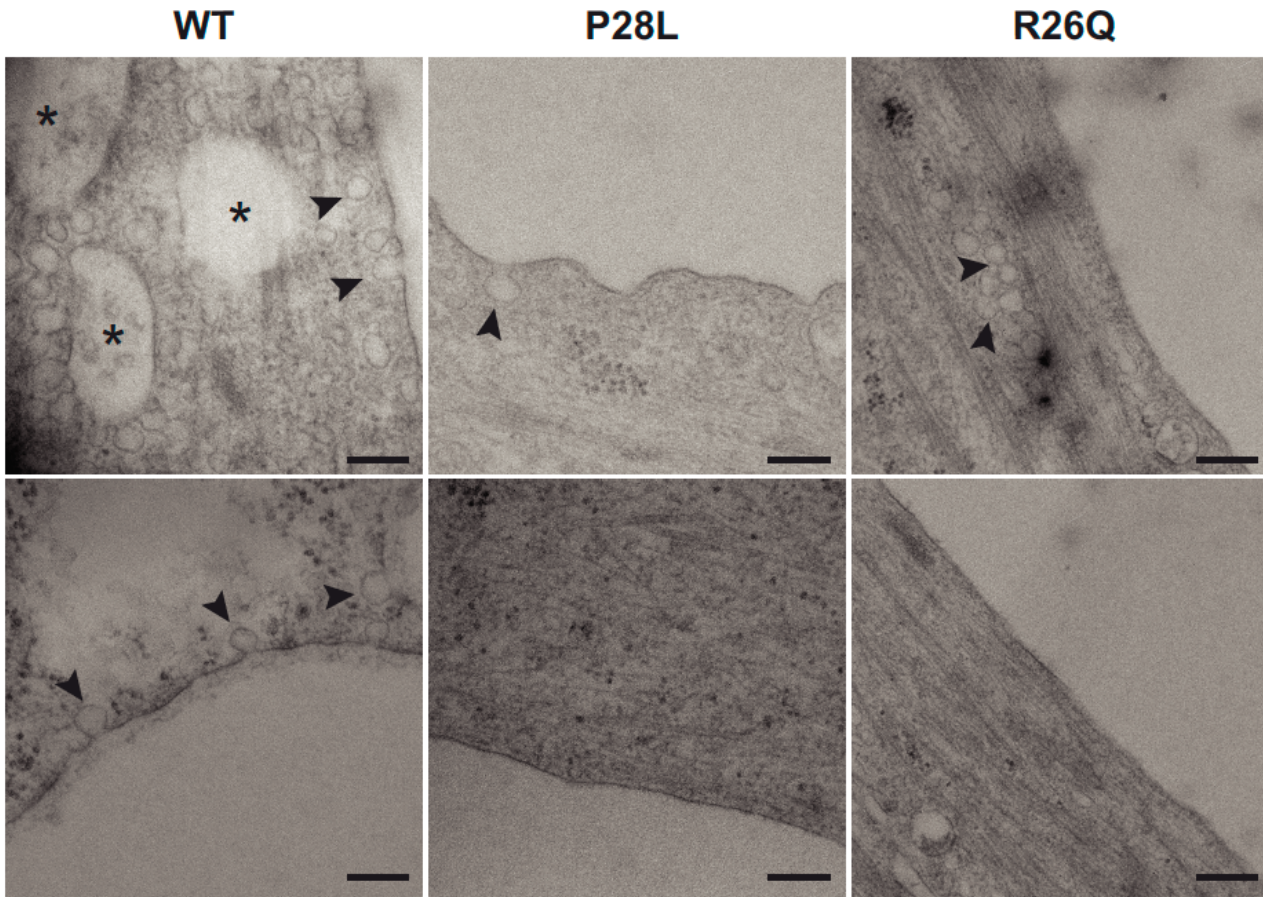
Performed by Melissa Dewulf

Supplementary figure 4:

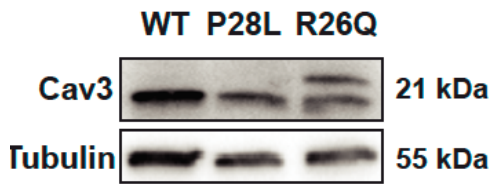
Experiments performed by Melissa Dewulf

Figure 1. Dewulf et al.

a



b



c

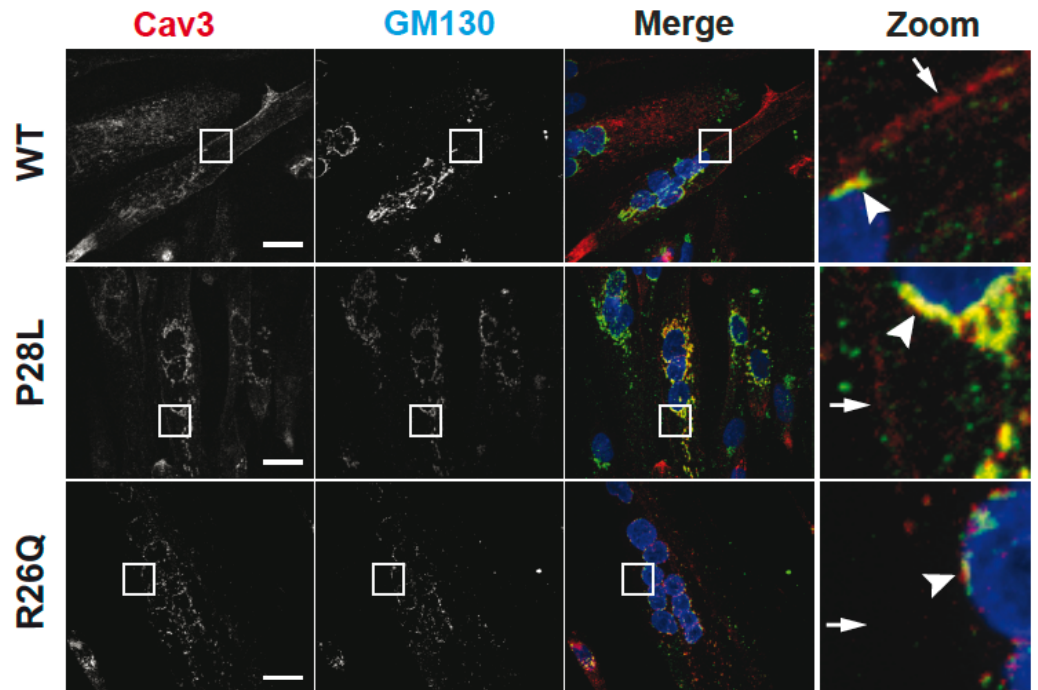


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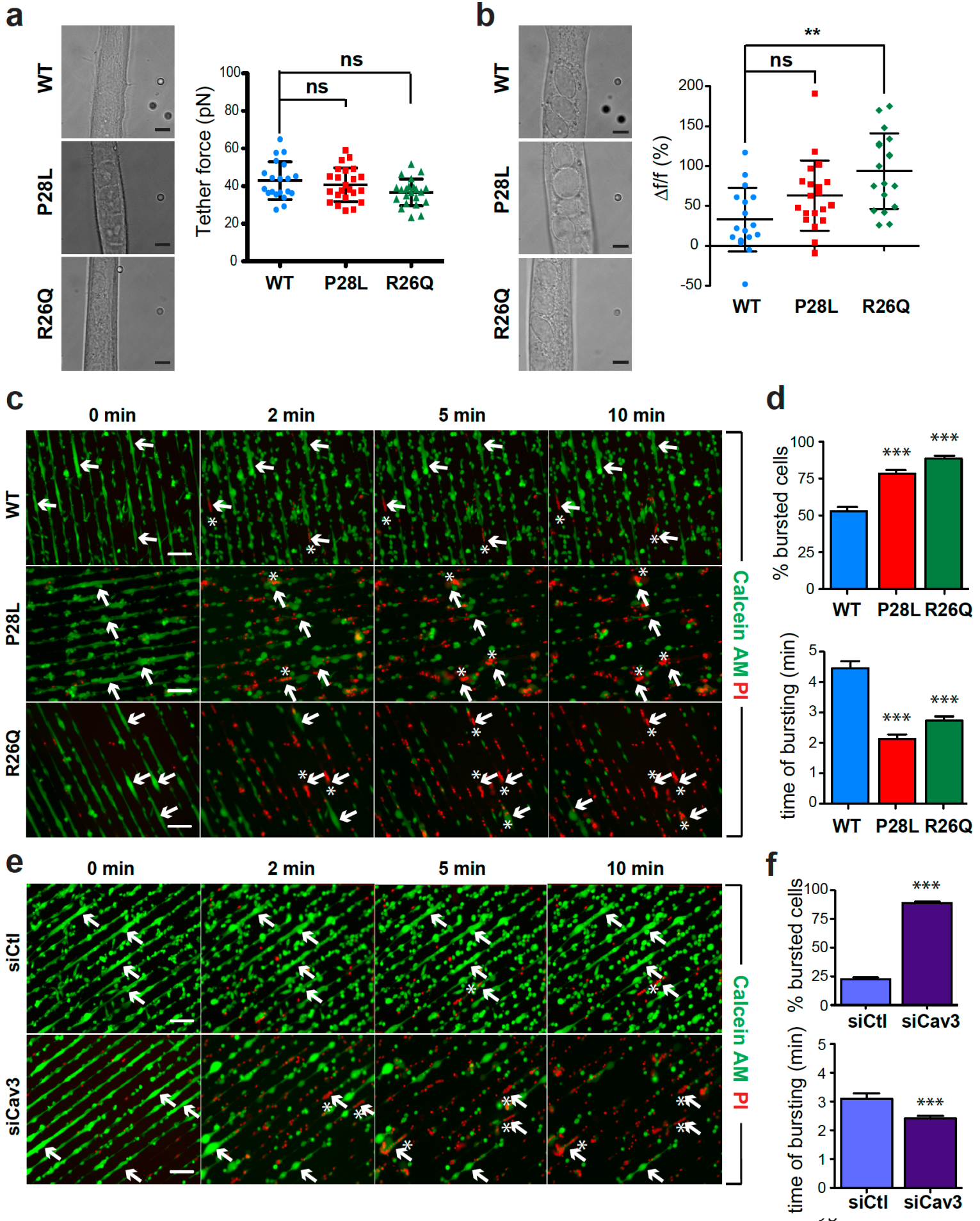


Figure 3. Dewulf et al.

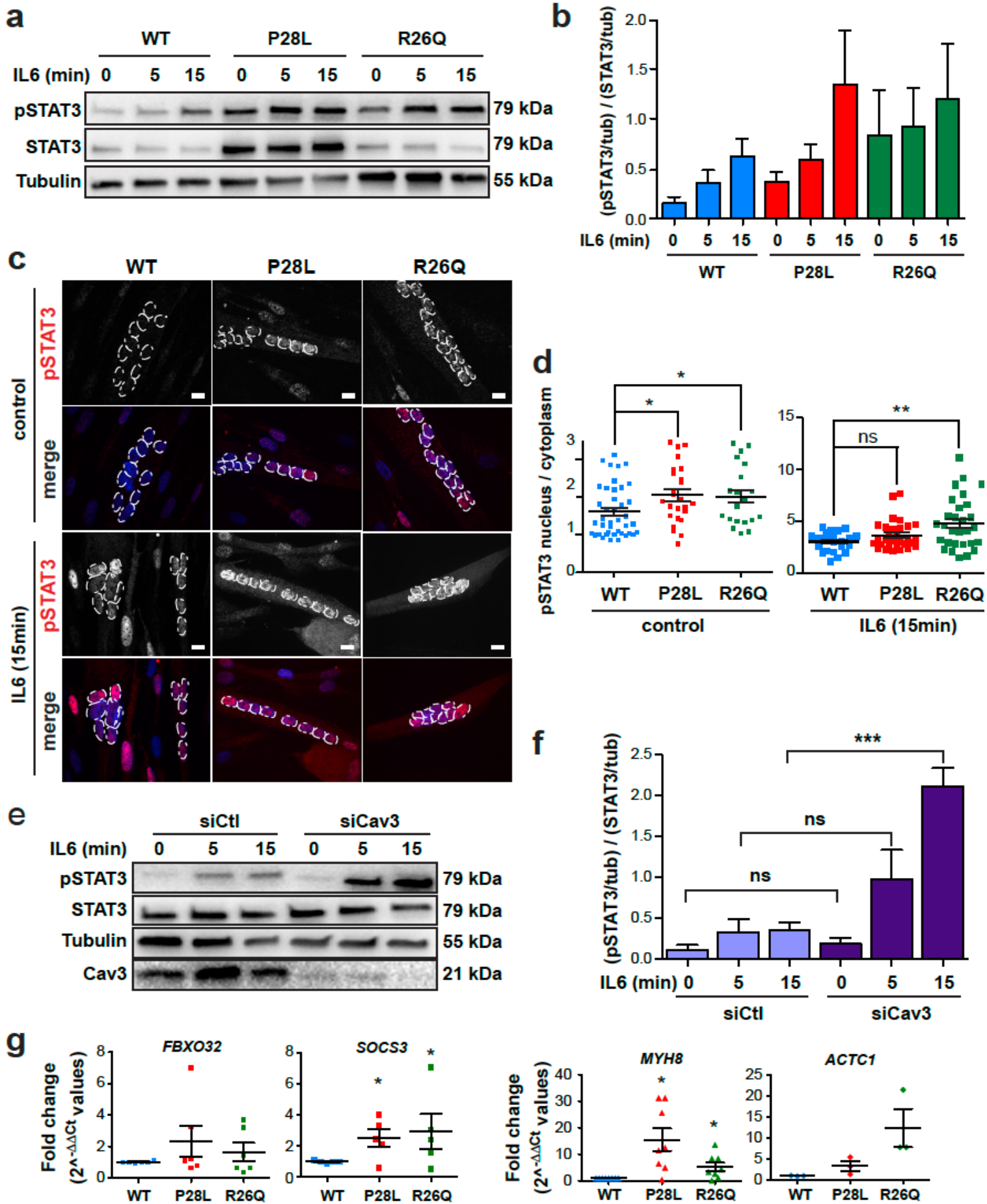


Figure 4. *Dewulf et al.*

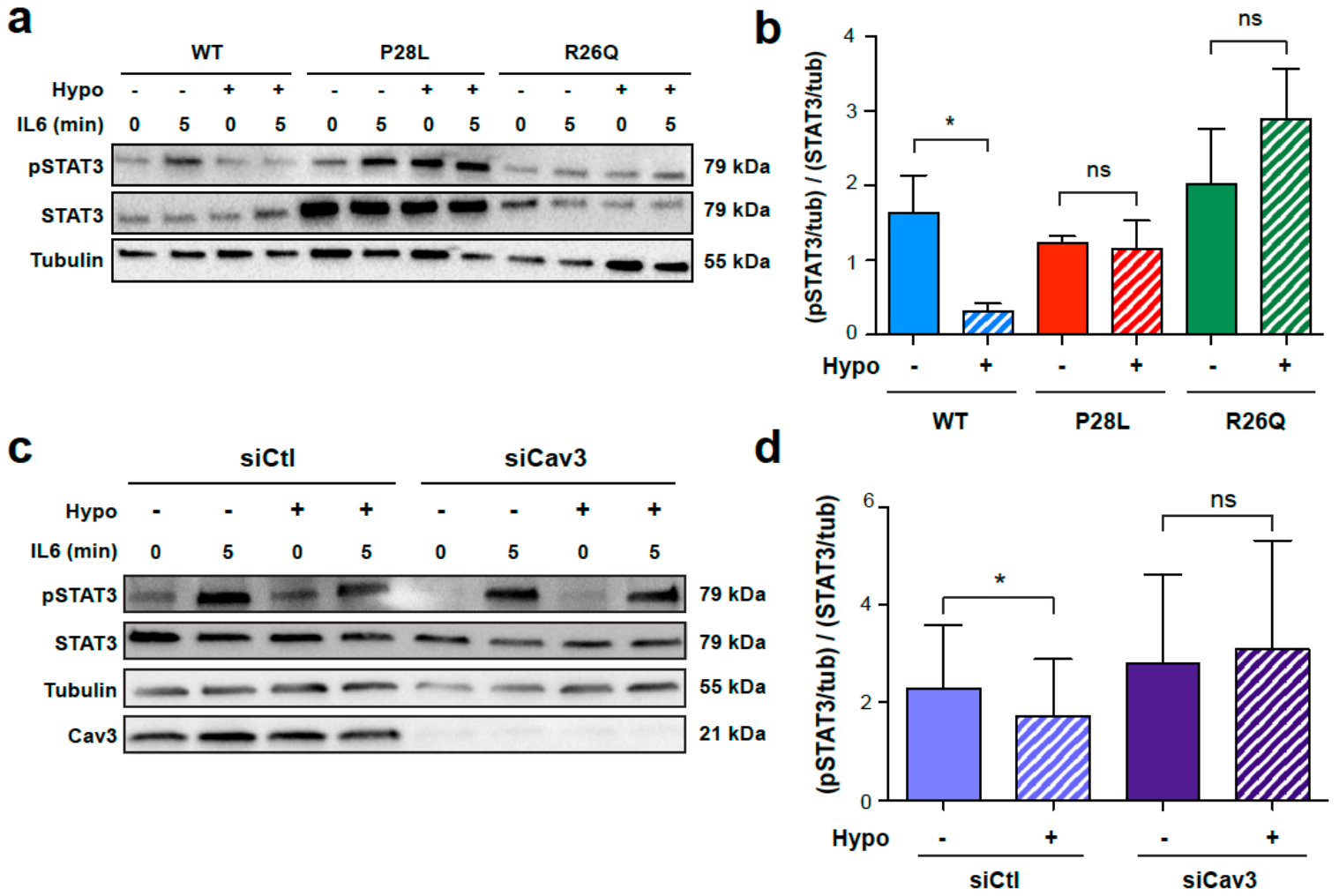
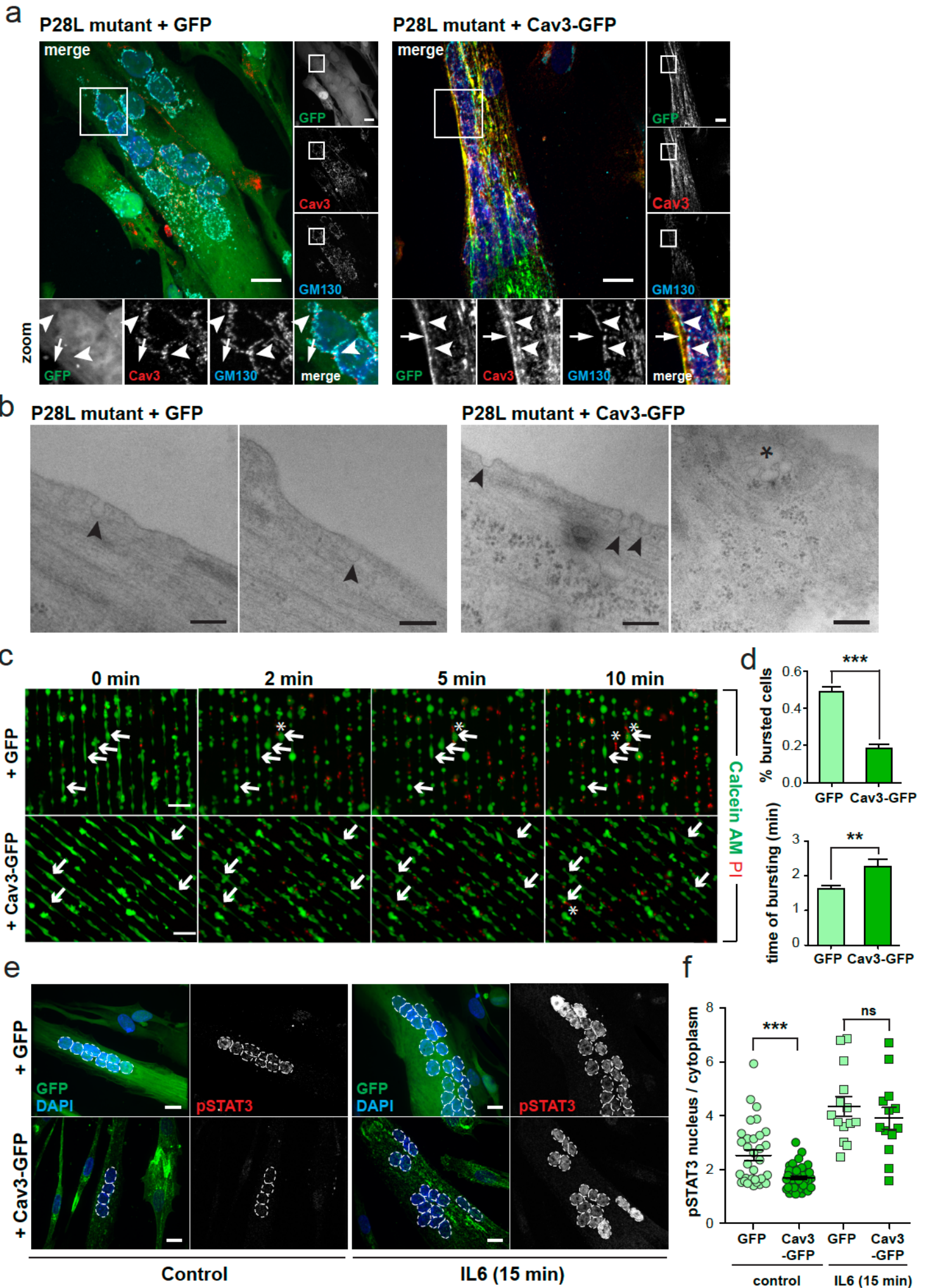
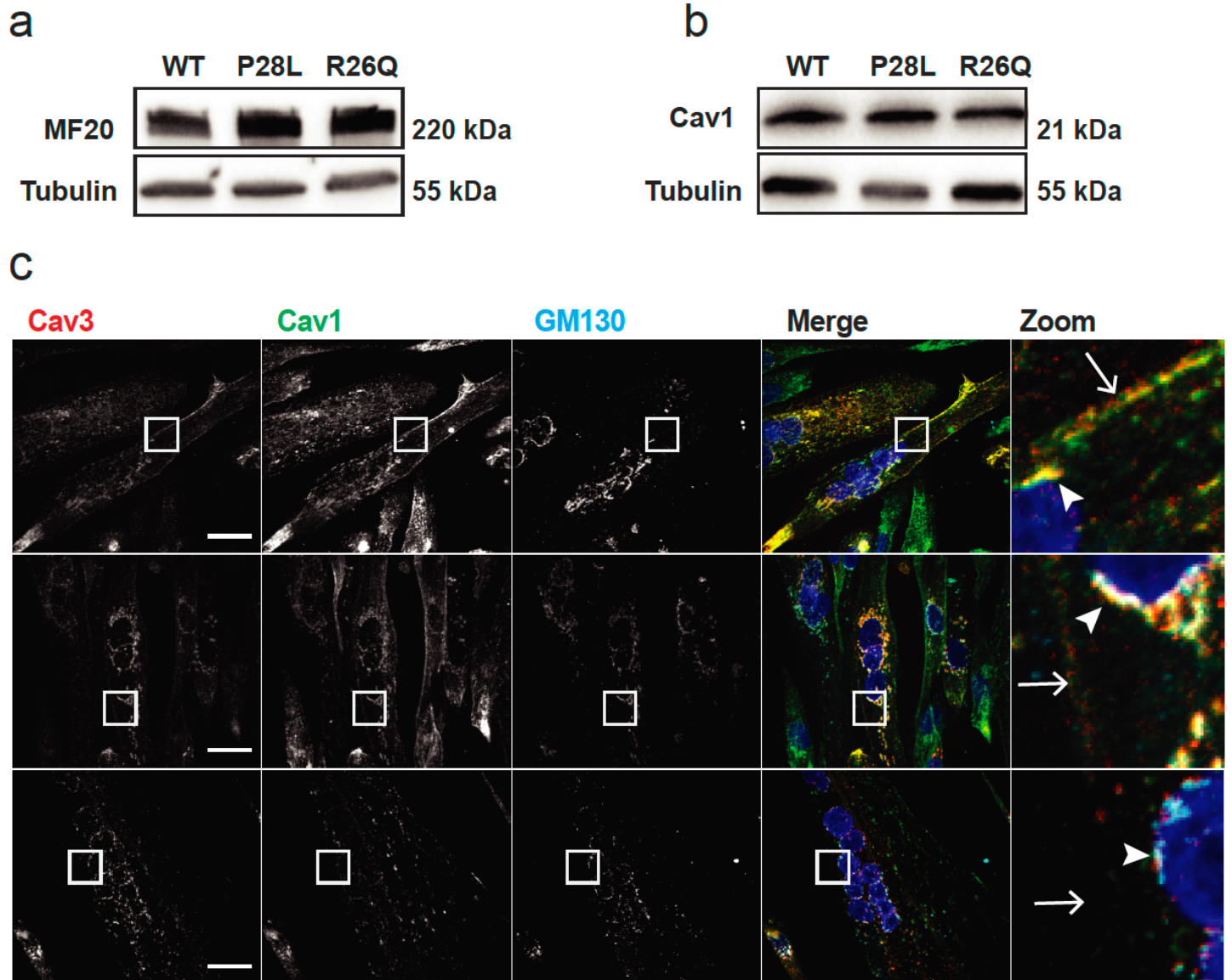


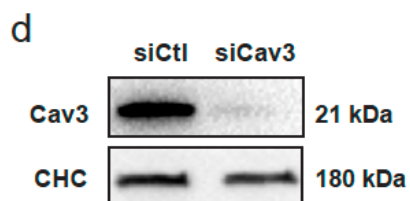
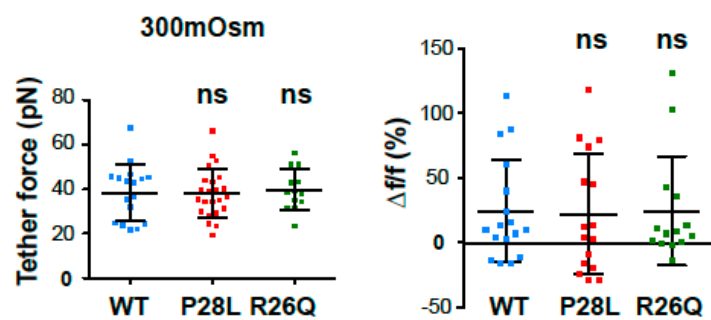
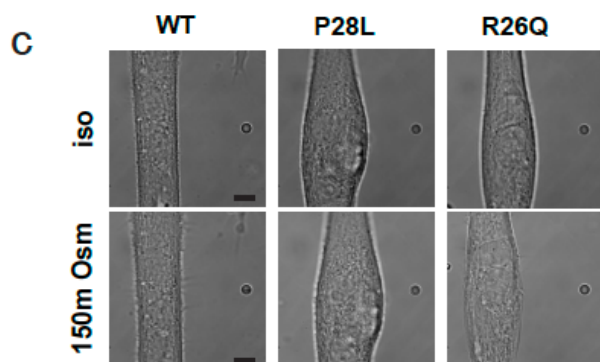
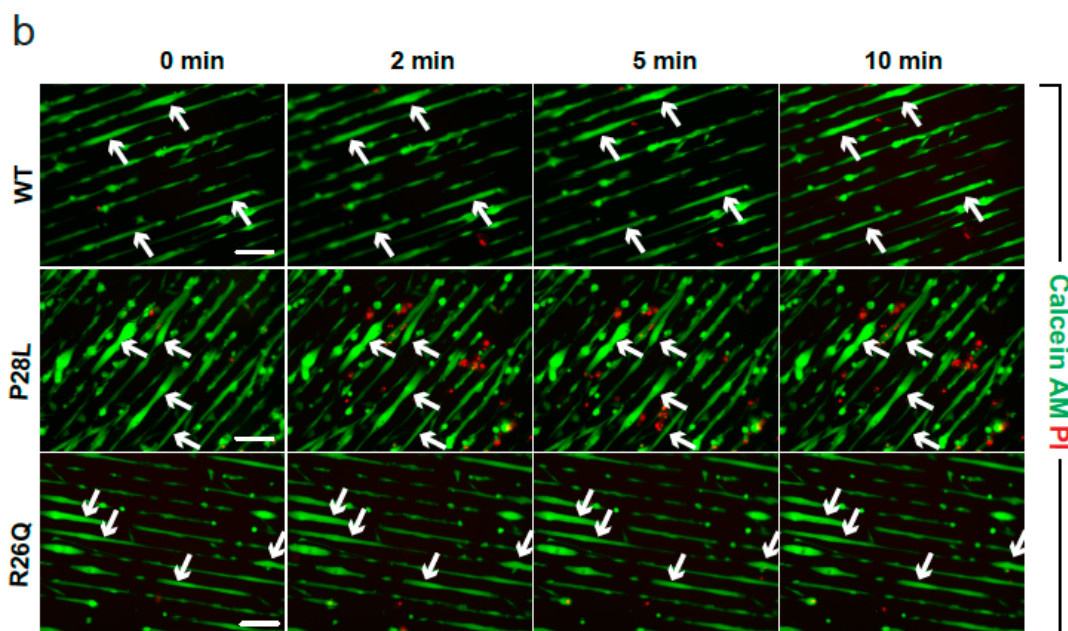
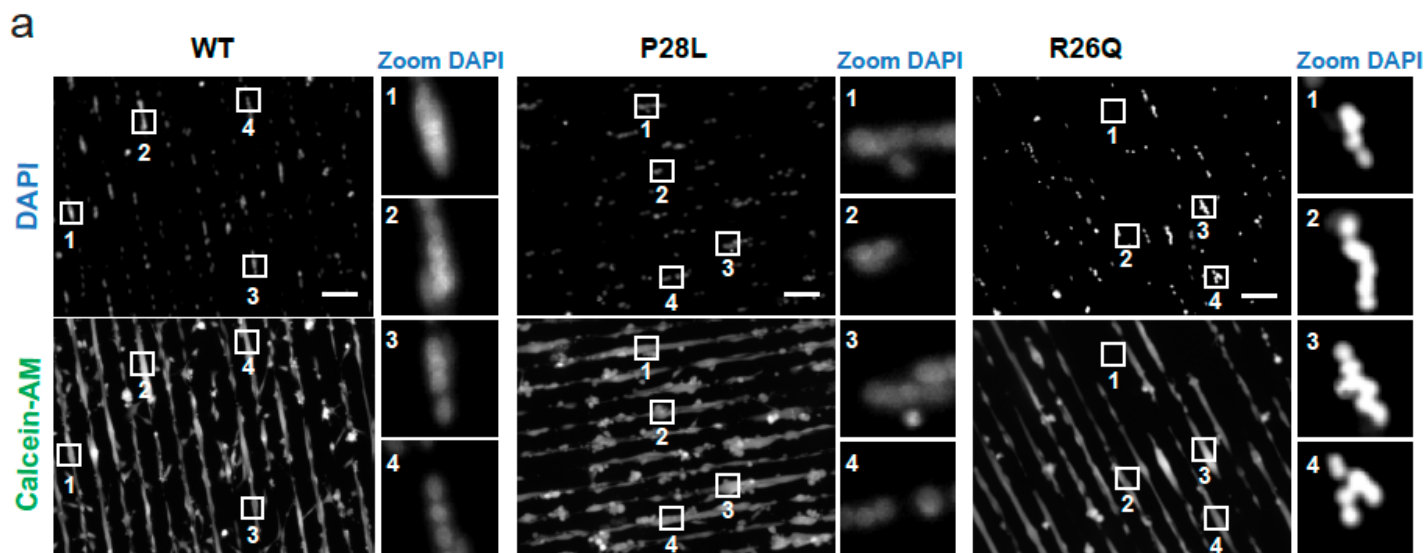
Figure 5. Dewulf et al.



Supplemental figure 1. *Dewulf et al.*

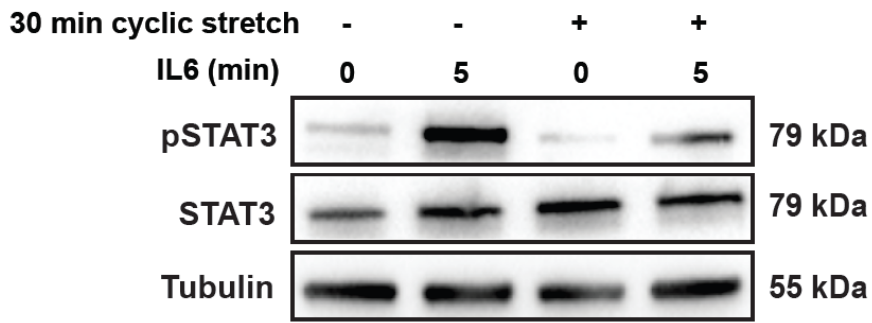


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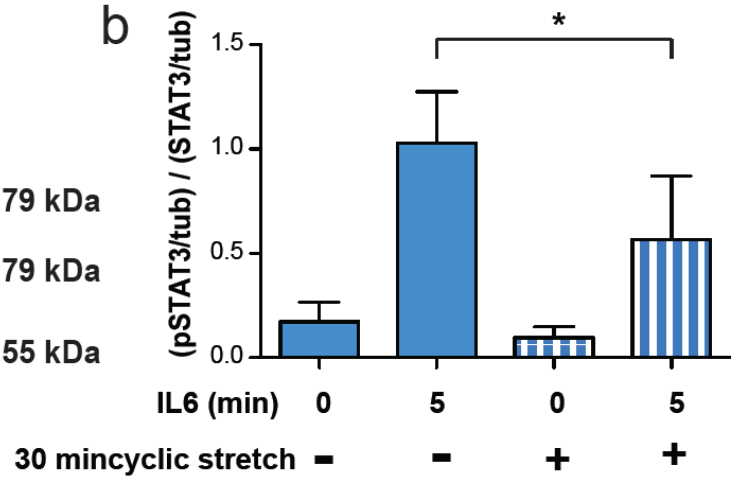


Supplemental figure 3. Dewulf et al.

a

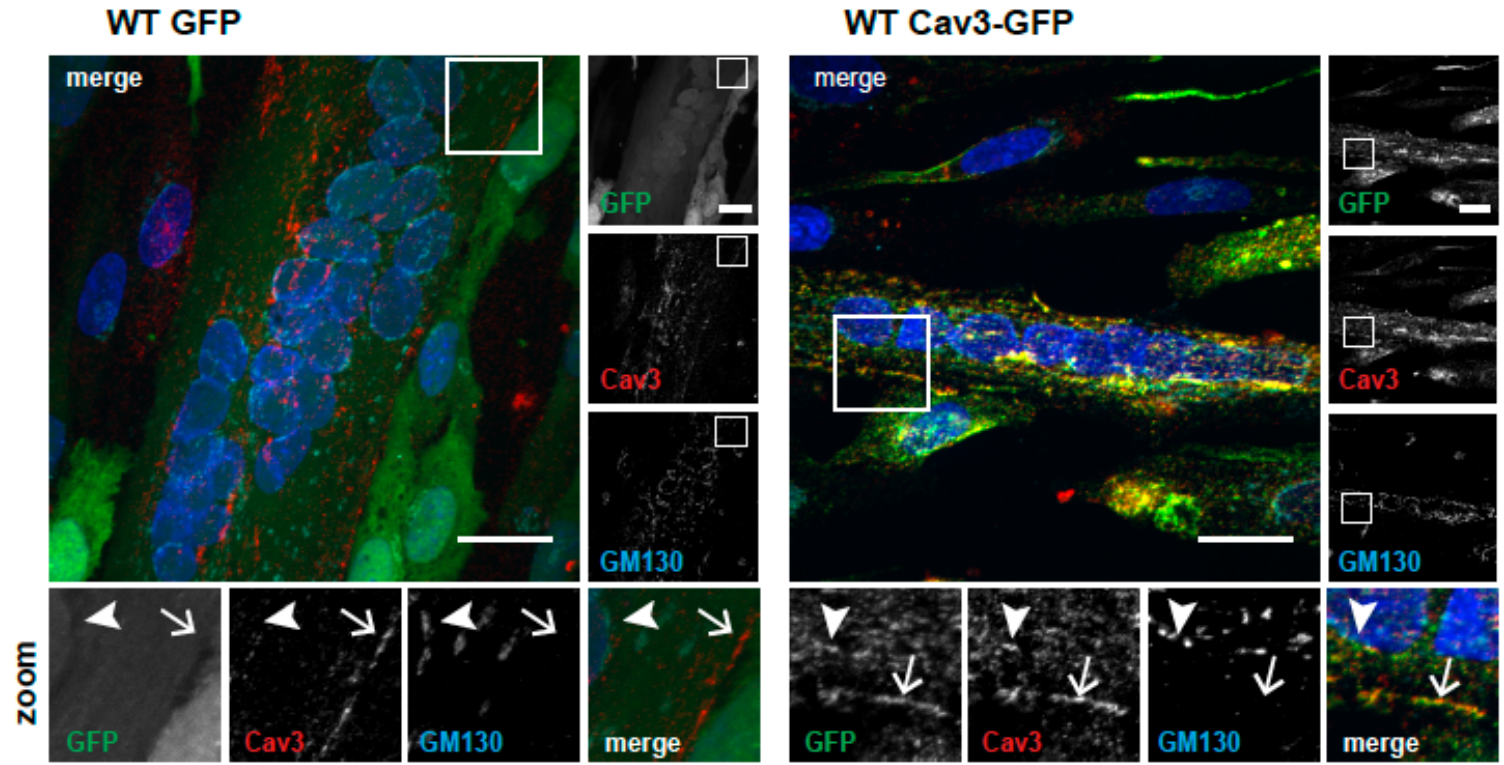


b

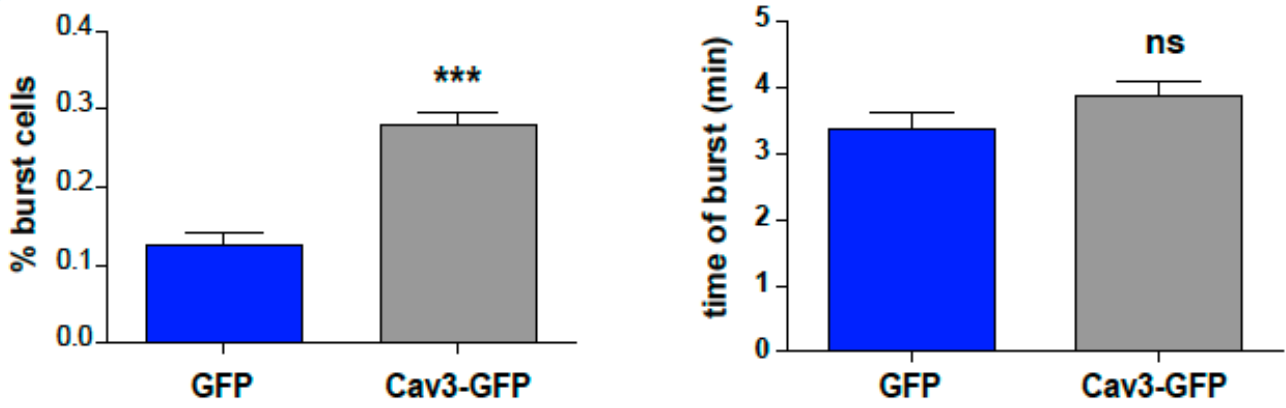


Supplemental figure 4. *Dewulf et al.*

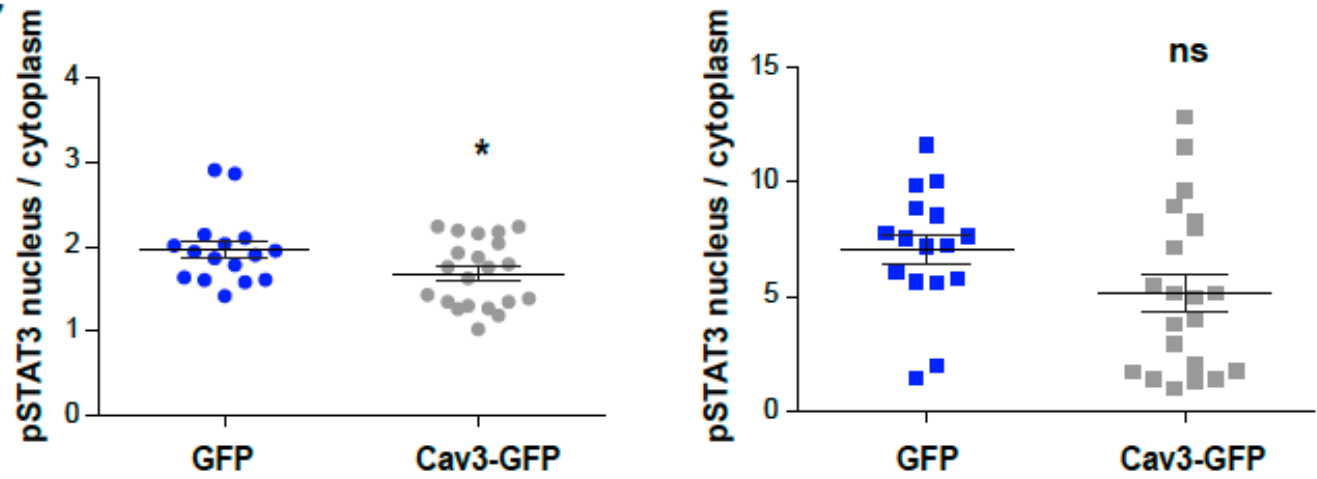
a



b



c



- DISCUSSION -

5 Discussion and perspectives

5.1 CAV3 mutations, retention in the Golgi complex and loss of caveolae at the plasma membrane

Although the CAV3 described mutations are distributed all along the Cav3 protein, it is intriguing that 95% of them lead to Cav3 retention in the Golgi complex. This is indeed the case in the Cav3 P28L and R26Q immortalized human myotubes that we have studied in this work. By studying the behavior of different mutated and truncated forms of Cav1, it has been shown that it was sufficient to mutate only one domain among all of the existing ones, to drive Cav1 retention in the Golgi complex. Furthermore, all Cav1 mutants had oligomerization and lipid association defects (Ren et al., 2004). Given the high degree of similarity between Cav1 and Cav3, it is reasonable to think that the same situation happens for CAV3 mutations. Accordingly, computational predictions show that the P104L mutation, which is associated with both LGMD-1C and RMD, could induce structural changes in all the protein domains of Cav3, despite its location in the intramembrane region (Deng et al., 2017). This suggests that conformational changes induced by point mutations may lead to oligomerization defects and impair the proper association of Cav3 with specific lipids and thereby proper export from the Golgi complex. Oligomerization defects remains to be proven, as it is in contradiction with the fact that most of Cav3 mutations are dominant negative and also lead to the retention of the WT form of Cav3, probably through oligomerization (Galbiati et al., 2000). Misfolding of the Cav3 protein could also lead to trafficking defect due to potential loss of interaction with trafficking partners. To test this hypothesis, comparative immunoprecipitation between WT and mutated Cav3 coupled to differential mass spectrometry analysis could be performed. The loss of interaction with potential partners along the trafficking route could either affect the Golgi complex exit, the delivery to the plasma membrane, or enhance Cav3 recycling back from the plasma membrane to the

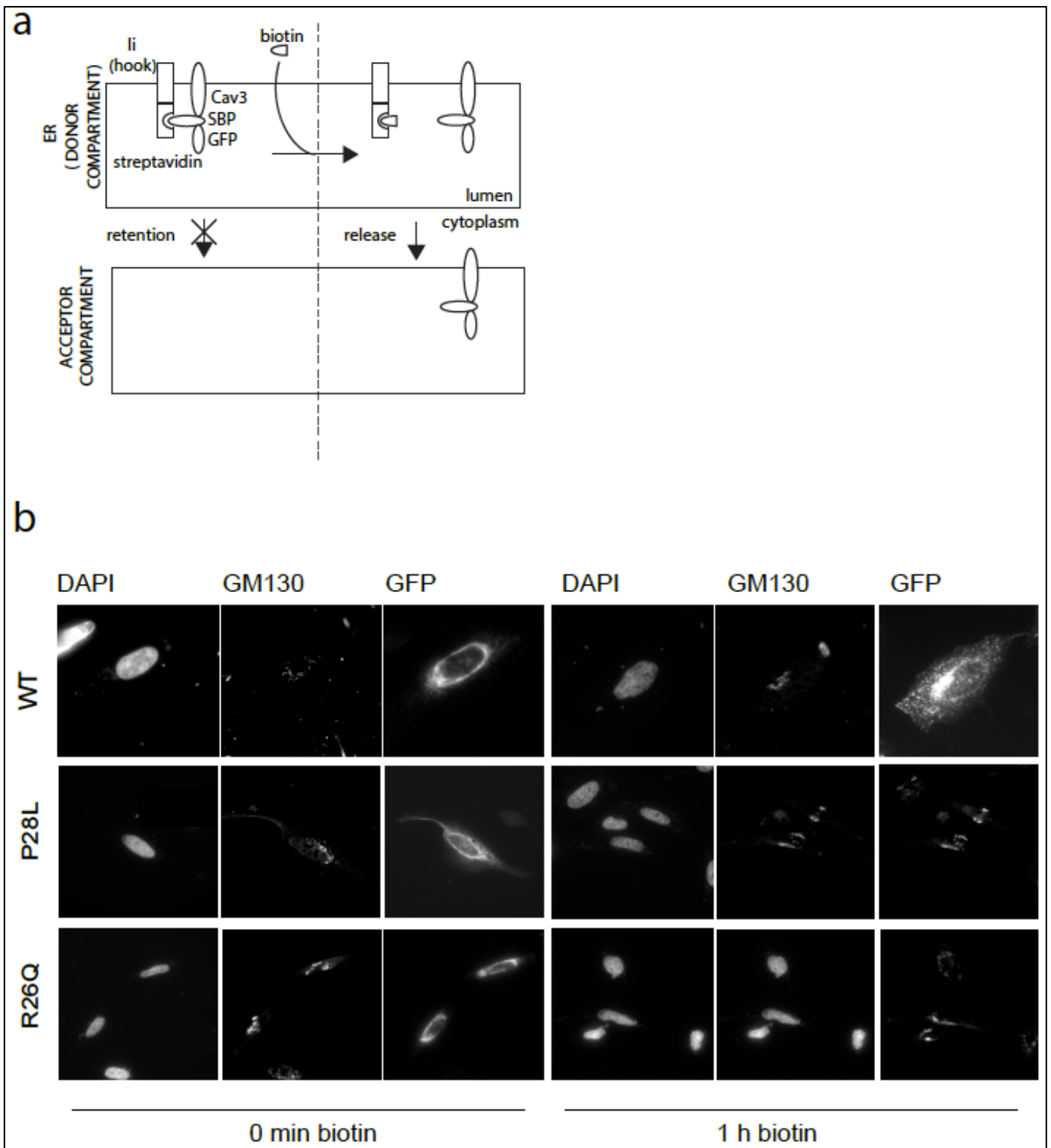


Figure 22. Synchronization of Cav3 trafficking.

(a) Schematic of the principle illustrates that the reporter is retained in the donor compartment, in our case, the ER, via its interaction with the hook (li). This interaction is mediated by the core streptavidin and the streptavidin binding protein (SBP). Release is induced by addition of biotin to allow trafficking of the reporter to its acceptor compartment. A fluorescent protein is fused to the reporter. In our case, the reporter is either Cav3 WT, Cav3 P28L, or Cav3 R26Q, fused with both an SBP and GFP (RUSH constructs).

(b) HeLa cells were transfected with either WT Cav3, Cav3 P28L or Cav3 R26Q RUSH constructs. 24h after transfection, cells were put in presence or not of biotin for the indicated time and fixed. Immunofluorescent labeling of RUSH constructs and Golgi marker GM130 in HeLa cells transfected with Cav3 WT-RUSH, Cav3 P28L-RUSH or Cav3-R26Q-RUSH analyzed by epifluorescence microscopy.

Golgi complex. To distinguish between these mechanisms, we could take advantage of the RUSH (Retention Using Selective Hook) system, which allows to synchronize and follow carefully the trafficking of a protein of interest upon simple biotin addition (Boncompain et al., 2012). We engineered RUSH constructs of the Cav3 P28L and Cav3 R26Q mutants and a pilot experiment shows trafficking from the ER to the plasma membrane of the WT form of Cav3, while Cav3 mutant trafficking stopped at the Golgi complex. Live microscopy would be needed to clearly determine whether Cav3 mutants are rapidly recycled or not (**Figure 22**).

In mutant myotubes, we showed that re-expression of Cav3 WT form, by locating itself at the plasma membrane, was sufficient to restore caveolae structures and rescue the observed phenotypes. Thus, it may be interesting to perform a screen for chemical compounds to identify drugs triggering the release of Cav3 from the Golgi complex in patient myotubes. Since mutated protein can adopt altered structures, reformed caveolae would be tested for their proper functionality.

We showed that CAV3 mutations lead to the loss of caveolae structures at the plasma membrane. What does happen to the other caveolae components in this context? Our immunofluorescence analysis show that Cav1 colocalizes in majority with Cav3 mutants in the Golgi complex, and a faint staining is also visible at the plasma membrane (see supplementary figure 1 of results). Cavin-1 seems to be more cytosolic (not shown), which is expected since there should not be any caveolae to be associated with at the plasma membrane. Cavin-1 could shuttle to the nucleus where it could regulate gene transcription since it was first described as a transcription factor. We did not carefully look at cavin-1 nuclear localization but it was observed in our laboratory that when cells undergo mechanical stress, releasing cavins in the cytoplasm, no increase in cavin-1 nuclear translocation was observed (Torrino et al., in revision), suggesting that higher levels of cytosolic cavin-1 would not enhance its transcription factor activity. We did not look at the other cavins but as cavin-1 is necessary for cavin oligomerization and

recruitment at the plasma membrane we can hypothesize that cavin-2, -3 and -4 are also mostly cytosolic.

5.2 Caveolae is required for plasma membrane integrity in muscle cells undergoing mechanical stress

In addition to our previous study (Sinha et al., 2011), we observed here that mutated myotubes could buffer membrane tension increase up to a 150 mOsm hypo-osmotic shock but not upon a higher hypo-osmotic shock (30 mOsm). Accordingly, we observe membrane integrity defects only in mutant myotubes subjected to a 30 mOsm hypo-osmotic shock. This suggests that muscle cells are particularly well adapted to mechanical stress and that acute/strong or prolonged exercise might induce a higher rate of muscle damage in the muscle tissue of CAV3-related MD patients. However membrane damage translates into muscle damage only if membrane repair is not able to compensate. As mentioned in the chapter 3.2.1.2 *Membrane repair*, Cav3 and caveolae have been shown to be tightly associated with membrane repair. Although the aim of this study was not focused on membrane repair, the membrane integrity assay we used could inform us about effective membrane repair after damage. To do this, we quantified the percentage of cells that were positive for both propidium iodide in the nucleus, which reports membrane damage, and calcein-AM, which indicates retention of the dye after rapid membrane repair. Our experiments were performed under hypo-osmotic medium for 10 min and therefore were not as accurate as monitoring dye influx after laser injury of muscle fiber. The latter showed that initial resealing is quite rapid but the recruitment of necessary proteins for proper membrane repair and remodeling takes more than 20 min (Marg et al., 2012). Although our experiments were performed for 10 min only, we observed yet a decrease of membrane repair events in both Cav3 mutants compared to WT. These data suggest that Cav3 mutations can affect membrane repair

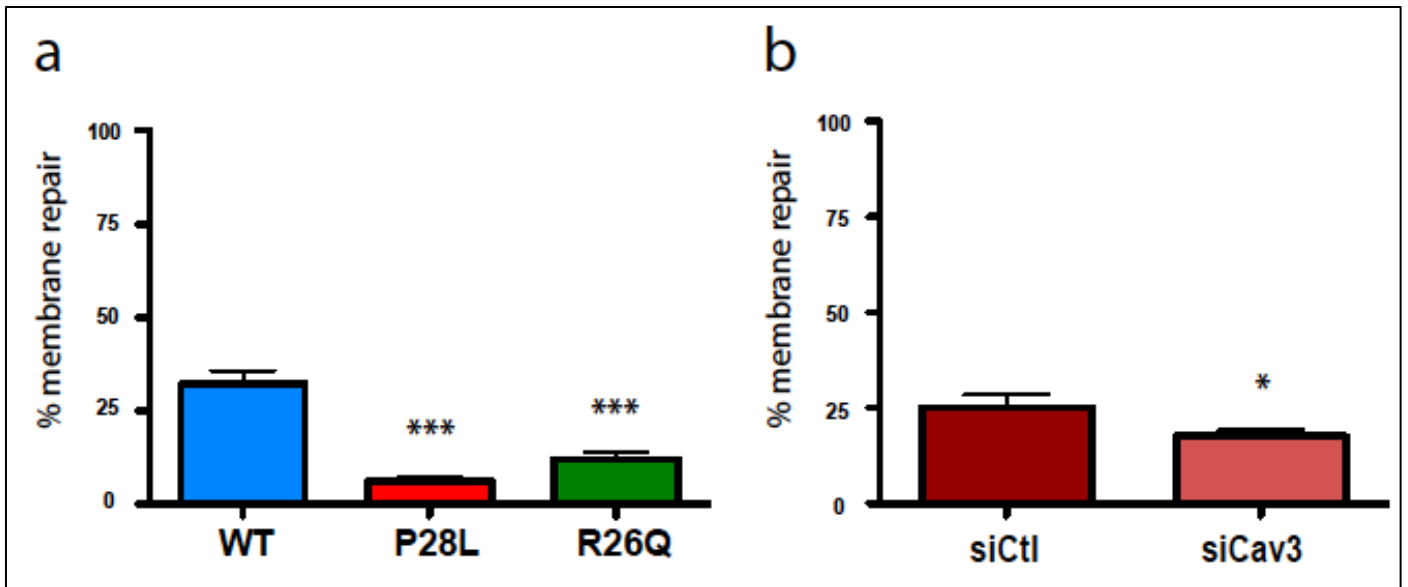


Figure 23. Membrane repair defect in Cav3 P28L and R26Q myotubes.

(a,b) Quantification of the percentage of membrane repair. WT, P28L and R26Q myotubes (a) or WT ctl (siCtl) or Cav3-depleted (siCav3) myotubes (b) were loaded with calcein-AM (green). The medium was switched with a 150 mOsm medium supplemented with propidium iodide (PI, red). Representative pictures were taken at the indicated times during hypo-osmotic shock. The percentage of membrane bursting corresponds to the percentage of cells positive for both PI and calcein-AM after 10 min of 30 mOsm hypo-osmotic shock. Scale bar = 10 μ m. Statistical analyzes with a two-tailed unpaired t test * $P < 0,05$ *** $P < 0,0001$

after injury in agreement with the published role of Cav3 in membrane repair (Cai et al., 2009; Hernandez-Deviez et al., 2008). Indeed, this membrane repair defect seems to be caveolae-dependent since Cav3 depletion induced decreased membrane repair in WT myotubes, and WT Cav3 expression restores partially membrane repair in P28L myotubes (**Figure 23**). Our observations are in accordance with the higher muscle damage seen in Cav3 R26Q expressing zebrafish kept in viscous medium (Lo et al., 2015). It might also explain the observation of variability in muscle fiber size and the presence of degenerating and regenerating fibers in muscle biopsies of CAV3-related MD patients (see *Table 1* of introduction).

Finally, Cav3 is not the only caveolae component that is associated with membrane repair. Indeed, EHD2 may be recruited at the site of injury together with annexin A1 and dysferlin, and stays there during the whole process of membrane remodeling (Marg et al., 2012). EHD2 seems to act independently from caveolae because its function was not modified in the context of the loss of caveolae induced by Cav3 mutations. Cavin-1 also may be necessary for membrane repair and would allow the anchorage of the membrane repair protein MG53, although this study doesn't allow to determine whether only cavin-1 is implicated or whether the whole caveolae structure is necessary.

5.3 Caveolin-3 and mechanical stress are negative regulators of the IL6/STAT3 pathway

Our results show that the IL6/STAT3 signaling pathway is negatively regulated both by Cav3 and mechanical stress. It would be interesting to know how this regulation is achieved at the molecular level, but also how these two means to inhibit the same pathway could be related.

As it has been previously described for other signaling pathways, this regulation could occur through direct interaction between Cav3 and key players of the IL6 pathway. In the

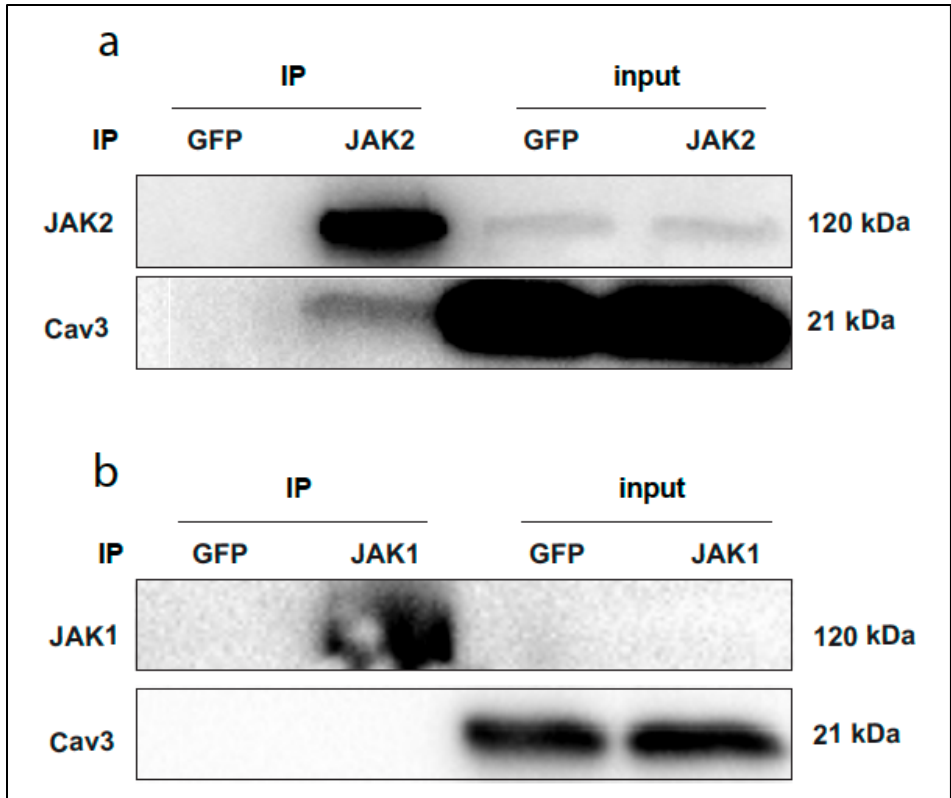


Figure 24. Cav3 interacts with JAK2.

(a, b) WT myotube cell lysates were incubated with anti-JAK1 (a) or anti-JAK2 (b) antibody to immunoprecipitate endogenous JAK1 or JAK2 and reveal co-immunoprecipitated endogenous Cav3. Immunoprecipitation of GFP serves as negative control.

case of the NO signaling pathway for example, this interaction is occurring through the caveolin scaffolding domain and a putative caveolin binding motif in the NO synthase (see chapter 2.3.3). Interestingly, we found by BLAST analysis that gp130, JAK1 and JAK2 have one or more putative CBM (not shown) and that gp130 has been shown to interact with Cav1 in a myeloma cell line (Podar et al., 2003). Furthermore, STAT3 can interact with Cav1 at the plasma membrane of fibroblasts, epithelial and endothelial cells, although this interaction does not impact STAT3 activity (Shah et al., 2002). To determine at which level this inhibition occurs, we should look at gp130 and JAKs activation state upon Cav3 depletion and mechanical stress. The interaction between Cav3 and its potential target can be established by different means, including colocalization assays, FRET (Förster resonance energy transfer), PLA (proximity ligation assay) and co-immunoprecipitations experiments. Preliminary experiments done in WT myotubes show that Cav3 co-immunoprecipitates with JAK2 but not with JAK1. However, this does not preclude any potential interaction between them (**Figure 24**). To demonstrate a direct interaction might be difficult, as caveolin purification has been very difficult to achieve due to high rate of protein aggregation. Nevertheless, the implication of the CSD could be investigated by using mimicking peptides (cavtratin/cavnoxin) or CSD mutated form of Cav3 (Bernatchez et al., 2005). If our hypothesis is correct, the use of these peptides should be sufficient to rescue the phenotype of IL6 hyperactivation at steady state. As mentioned before, one way to release a larger pool of “free” Cav3 into the plasma membrane is to apply a mechanical stress (Sinha et al., 2011). We could thus hypothesize that the observed effect of mechanical stress on IL6 pathway may be due to a higher amount of released free Cav3 available at the plasma membrane following the immediate disassembly of caveolae. Hypo-osmotic shock is a severe stress for the cell and we cannot exclude that other mechanisms may be involved but our data showing a decreased inhibition of the IL6 pathway upon mechanical stress in Cav3 depleted cells strongly suggest that Cav3 is an important player in this process (see *Figure 4* of the paper).

An interaction with Cav3 through its CSD shouldn't explain why the IL6 pathway is already overactivated at steady state in mutant myotubes. Indeed, Cav3 should be associated with caveolae in WT cells and would thus not be able to interfere with the pathway. It has been observed for a long time that even in resting condition, both invaginated and flat caveolae can be observed (Rothberg et al., 1992). Regardless how caveolae flatten out at steady state, it should lead to the release of a fraction even minor of Cav3 in the plasma membrane, which should be visible by EM coupled with immunolabelling of Cav3. Cav3 being totally absent from the plasma membrane of mutant myotubes, this could partially explain the fact that the IL6 pathway is hyperactivated in these cells. Another important player in the regulation of this pathway, the negative regulator SOCS3, may also not function properly in these myotubes. Indeed, a recent study showed that SOCS3 needs cavin-1 for its targeting to the plasma membrane and its function (Williams et al., 2018). Although the mechanism of cavin-1 induced SOCS3 plasma membrane targeting remains unknown, the initial localization of cavin-1 at the plasma membrane may be crucial. In Cav3 mutant myotubes, SOCS3 gene expression is increased, but cavin-1 is mainly cytosolic, suggesting that SOCS3 may not get properly targeted and cannot inhibit the pathway.

What consequences can arise from the abnormal hyperactivation of the IL6 pathway in skeletal muscle from patients bearing CAV3 mutations? In these cells, we showed that the expression of muscle regeneration-associated genes *MYH8*, *ACTC1* and *SOCS3* was upregulated. Their expression could be upregulated by STAT3 activation but we need further experiments to establish this link in a direct manner, such as the use of pharmacologic compounds inhibiting STAT3 activity or STAT3 silencing. Furthermore, if IL6 is capable of positively regulating its own expression (see chapter 1.3.4.2), the chronic autocrine secretion of the cytokine could also stimulate differentiation and fusion of satellite cells involved in muscle regeneration. At long term, chronic activation of the IL6 pathway could lead to satellite cell exhaustion, resulting in muscle atrophy. Other

genes that are often associated with muscle atrophy have also been shown to be upregulated when STAT3 is overactivated, such as *FBXO32*, *Atrogin1*, or *C/EBP δ* (CCAAT-enhancer-binding proteins). *C/EBP δ* is a transcription factor controlling the expression of myostatin, a major inducer of proteolysis (Bonetto et al., 2011). In our system however, we could not observe any difference between mutant and WT myotubes for *FBXO32* expression.

5.4 Caveolin-3 mutations are associated with different clinical phenotypes

As discussed in chapter 3.1 *CAV3 mutations and muscle diseases*, *CAV3* mutations can induce four distinct clinical phenotypes of skeletal muscle diseases. Some symptoms and some features of muscle biopsies are however common between the different phenotypes: high serum creatine kinase (CK) levels signaling muscle cell lysis, muscle hypertrophy, variability of muscle fiber size and increased central nuclei. In this chapter, I will discuss how the results we obtained by studying human Cav3 P28L and Cav3 R26Q myotubes can be linked to the clinical features listed above.

High CK levels and variability of muscle fiber size can both result from impaired muscle integrity, and increased muscle damage, which was already discussed in the chapter 5.2 *Caveolae contribute to plasma membrane integrity in muscle cells undergoing mechanical stress*. Increased Cav3-related muscle damage could also be responsible for increased central nuclei. Indeed, it has been shown that regenerating muscle fibers had more central nuclei, although it is not clear whether it is an intermediate state or a permanent feature (Maxwell et al., 1984). Cav3 could also perturb nuclei localization by disturbing the DGC complex and desmin organization. This remains to be proven since the effect of Cav3 loss on the stability and function of the DGC is still unclear.

The observation of muscle hypertrophy in patients could be partially explained by the overexpression of muscle regeneration associated genes. Indeed, although regenerating

muscle fibers can be a consequence of muscle damage, the expression of *MYH8*, *ACTC1* and *SOCS3* could also be controlled by STAT3, as it was suggested before (Bonetto et al., 2011). This remains to be directly proven by using STAT3 inhibitors or STAT3 depletion, but if this is correct, the chronic activation of STAT3 could enhance muscle fiber regeneration and at long term potentially lead to muscle hypertrophy.

However muscle atrophy and hypertrophy both rely on the activity of many different pathways. Similarly, a lack of Cav3 expression has been associated with both decreased and increased myoblast fusion, also depending on different pathways (Ohsawa et al., 2006; Volonte et al., 2003). It is likely that to determine the implication of Cav3 in muscle atrophy or hypertrophy will be complex and will need further investigations.

One important point to raise here is that even though many other signaling pathways have been associated with caveolae before, only few of them have been considered through caveolar mechanosignaling. Notably, several pathways are associated with mechanical stress, such as the ERK and the Akt pathway (Kawamura et al., 2003; Sedding et al., 2005). It would thus not be surprising that Cav3 and caveolae could be involved in their sensitivity to mechanical stress, as it is the case for the IL6 pathway.

Although they have common features, it is still surprising that *CAV3* mutations are associated with as much as four different clinical phenotypes. Indeed, *CAV3* mutations are numerous, yet they all lead to retention in the Golgi complex. We would thus expect that all *CAV3* mutations would translate into only one clinical phenotype, like many other mutated proteins associated with muscle diseases. This difference of clinical phenotypes observed between the different *CAV3* mutations could be due to the difference of genetic background between individuals, although it is very difficult to prove. It could also be that depending on the mutation, the protein could be expressed at different levels and have different conformations. This could induce changes in the interaction with potential partners, such as signaling molecules, and would regulate differently some signaling pathways. This hypothesis was proposed when Brauers et al. showed a

difference in the regulation of some growth factor signaling (nerve growth factor and epidermal growth factor) by the two CAV3 mutations we investigated on in our study: P28L and R26Q (Brauers et al., 2010). Furthermore, one mutation can be associated with several clinical phenotypes, even within the same individual, highlighting that a strict genotype-phenotype association does not exist in caveolinopathies.

Many progress have been made since the first identification of muscle disorder associated CAV3 mutation, but the underlying molecular aspects remain poorly understood. The recent development of new tools to study caveolinopathies, such as the generation of human muscle cell lines expressing endogenous CAV3 mutations, CRISPR technology or the application of mechanical stress combined with microscopy, to name only a few, should help providing a better understanding of the molecular mechanisms associated with these mutations. By extension, these tools could also be of use in the context of other caveolae components associated diseases such as lipodystrophy, atherosclerosis or cancer.

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- ANNEX-

ANNEX



The caveolae dress code: structure and signaling

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Abstract

Over the past decade, interest in caveolae biology has peaked. These small bulb-shaped plasma membrane invaginations of 50–80 nm diameter present in most cell types have been upgraded from simple membrane structures to a more complex bona fide organelle. However, although caveolae are involved in several essential cellular functions and pathologies, the underlying molecular mechanisms remain poorly defined. Following the identification of caveolins and cavins as the main caveolae constituents, recent studies have brought new insight into their structural organization as a coat. In this review, we discuss how these new data on caveolae can be integrated in the context of their role in signaling and pathophysiology.

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The caveola robe

Caveolins

It took almost 40 years after caveolae were first visualized by electron microscopy (EM) in the 1950s to identify the caveolar protein components [1,2]. Caveolin-1 (Cav1) was identified in 1992 [3,4] followed by the two others homologues caveolin-2 (Cav2) [5] and the muscle specific caveolin-3 (Cav3) [6,7]. The three caveolin isoforms contain a family signature constituted by a single stretch of eight amino acids ⁶⁸FEDVIAEP⁷⁵ localized in the N-terminal cytosolic oligomerization domain (Figure 1a) [7].

Cavins

Although Cav1 and Cav3 were initially thought to be necessary and sufficient for caveolae morphogenesis [8],

several studies in the past decade have uncovered additional constituents. Thus, the identification of the cavin protein family brought precious insights into caveolae ultrastructure and assembly opening new avenues for better understanding the cellular functions of this intriguing organelle. The assembly of a *bona fide* caveola requires both Cav1 and cavin-1 (also called PTRF) [9,10]. In mammals, cavin-2 (SDPR), cavin-3 (SRBC) and the muscle-restricted cavin-4 (MURC) complete this four-member family, which has emerged as essential to caveolae formation and functions (Figure 1b) [11–13].

Purified cavins when added on phosphatidylserine (PS) enriched liposomes or when overexpressed in mammalian cells induce membrane tubulation, leading to the assumption that cavins may play a role in the initiation of the caveola invagination [11,14,15**]. Accordingly, cavin-1 depletion results in loss of caveolae while caveolae are not morphologically detectable in the prostate cancer PC3 cell line and in the nematode *Caenorhabditis elegans*, which both express Cav1 but not cavin-1 [9,16]. Nevertheless, it was reported that caveolae could be assembled in *Escherichia coli* independently from cavin-1 [17].

Accessory proteins

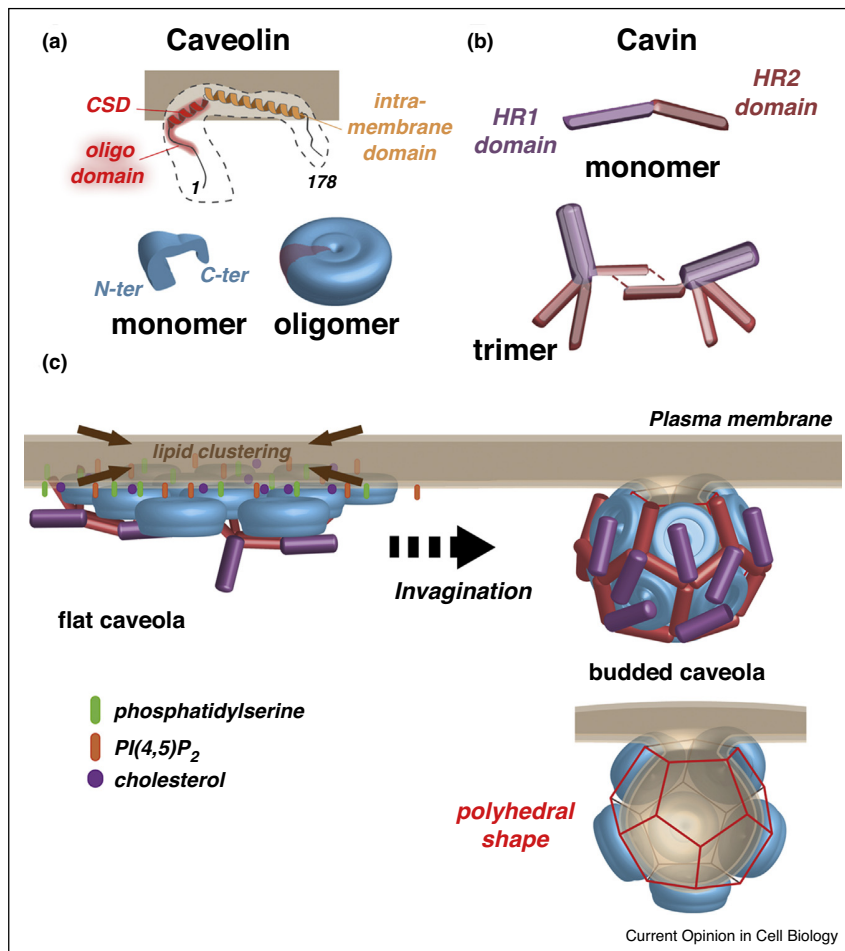
Several non-essential proteins have also been involved in caveolae biogenesis. PACSIN-2, also called Syndapin2, is the only F-BAR protein representative, proteins regulating membrane curvature, that has been involved in caveolae morphology [18,19]. The dynamin-2 GTPase and the dynamin-like ATPase EHD2 have been localized at the neck of caveolae [20,21]. EHD2 has been shown to stabilize caveolae by controlling their dynamics and association with the actin cytoskeleton [22]. Dynamin-2 however is not present in all caveolae and whether it plays a role in caveolae scission similarly to its classical role in clathrin-dependent endocytosis remains to be established.

A tailored coat

Caveolins bind lipids and organize membrane nanodomains

The assembly process is initiated by the export of caveolin-enriched vesicles from the Golgi apparatus to the plasma membrane [23]. Little is known about this first step except for a critical role of lipids and particularly cholesterol, which is essential for both caveolin oligomerization at the Golgi apparatus and caveola invagination at the plasma membrane [3]. Cav1 binds cholesterol with a

Figure 1



Putative model of caveolar coat assembly and organization.

(a) Schematic model of Cav1 topology. Cav1 is inserted into the plasma membrane through the caveolin scaffolding domain (CSD; red), an amphipathic helix part of the oligomerization domain (diffuse red), and through a second amphipathic helix, the intra-membrane domain (orange). Based on Cav3 ternary structure [108], Cav1 monomers may assemble as a disk-shaped oligomer with the C-terminal part oriented toward the center. **(b)** Cavins exhibit two helical rich domains, HR1 and HR2, that may form coil-coiled structures [14]. Cavins, through interaction with the HR1 domain, can form trimers consisting of either three cavin-1 or two cavin-1 associated with one cavin-2 or one cavin-3 protein. The cavin-1 isoform could be responsible for a more complex assembly through the coiled-coil domain 2 (cc2) sequence in the HR2 domain [15]. **(c)** At the plasma membrane, Cav1 oligomers cluster specific lipids such as cholesterol, PI(4,5)P₂ and phosphatidyl serine involved in the recruitment of cavin trimers. This is followed by caveola invagination, a process not completely understood. It has been recently suggested that the overall architecture of the caveolar coat made of caveolins and cavins would best fit with a polyhedron structure [15,30]. In this model, Cav1 oligomers position on each pentagonal face and cavin complexes align with the vertices and cover the Cav1 oligomers.

1:1 stoichiometry [24,25], probably through a cholesterol recognition/interaction amino acid consensus (CRAC) motif (⁹⁴VTKYWFYR¹⁰¹) located in the vicinity of the plasma membrane [26]. Caveolin oligomers trigger the clustering of specific lipids thereby constructing specialized lipid nanodomains at the plasma membrane [27]. They include sphingolipids (sphingomyelin, GD3 and GM1 gangliosides), phospholipids such as PS and phosphoinositides such as phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂).

Cavins are recruited to caveolin-induced lipid nanodomains

Interestingly, PS and PI(4,5)P₂ were recently involved in caveola formation through electrostatic interactions between the negatively charged headgroups and two specific domains of cavins (HR1 & HR2) [9,12,14,15^{••},28]. Thus, cytosolic cavins form higher-order heterotrimers consisting of either three cavin-1 or two cavin-1 with one cavin-2 or one cavin-3, through their HR1/cc1 coiled-coil domain, that further polymerize

upon association with assembling caveolae (Figure 1c) [14,21,29].

Flat versus curved: the interplay between caveolins and cavins

At the plasma membrane cavins and caveolins invariably form characteristic stripes surrounded by a proteinaceous crescent made of globular proteins whose identity remains elusive (Figure 2). To date, it is still unclear whether caveolae assemble as flat structures that will then bud inward and produce the typical stable cup-shaped caveolae or if pre-formed caveolae emanating from intracellular compartments most likely the trans-Golgi network fuse with the plasma membrane.

Recent EM and X-Ray crystallography studies revealed that the characteristic striated coat that is observed on the outer cytoplasmic side of caveolae may be organized by cavins rather than caveolin oligomers alone as originally proposed [14,15^{**},21,29,30^{**}]. These striations are observable on deep etch electron micrographs of caveolae

presenting various degrees of invagination from flat to fully budded (Figure 2).

It has been estimated that 150–200 Cav1 monomers associate with 50–60 cavins (≈ 15 –20 trimers) to form a caveola [15^{**},21,29,31]. The overall architecture of caveolae was recently proposed to fit with a polyhedron most likely a dodecahedron structure formed by cavin complexes aligned with the vertices but also covering the caveolin oligomers positioned on each pentagonal face (Figure 1c) [15^{**},30^{**}]. It is however difficult to visualize a dodecahedron organization when observing caveolae *en face* on deep-etch electron micrographs, and further efforts will be needed to validate this model with proteins which have been purified from more physiological systems than insect cells and bacteria.

Signaling regulation through direct interaction with Cav1

Over the years, caveolae have been associated with various physiological and pathological contexts in relation with their cellular functions in lipid homeostasis, signal transduction, endocytosis and transcytosis. If some debate still exists [32], early consensus suggested that caveolae could regulate cellular signaling by organizing specific signaling platforms at the plasma membrane [33].

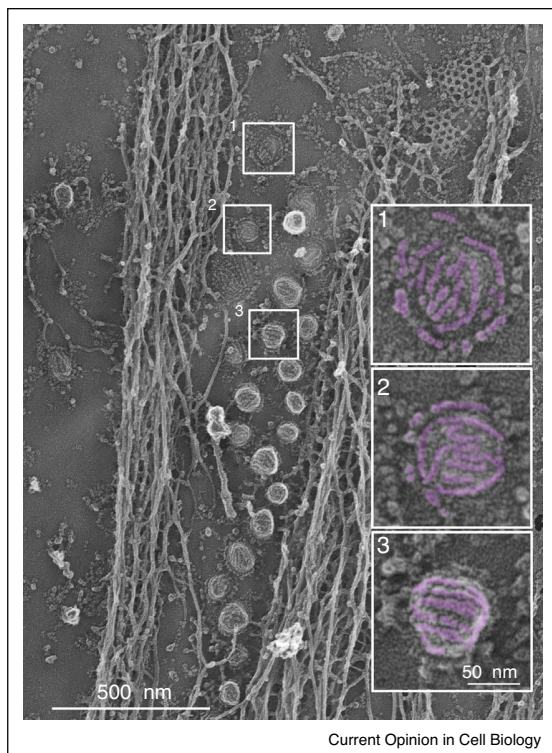
Thus, a broad variety of growth factor and signaling receptors, kinases, enzymes and other signaling molecules have been localized into caveolae and/or co-immunoprecipitated with Cav1 including but not limited to eNOS [34], the insulin [35], EGF [36], TGF- β [37^{*}] and P2X7 [38] receptors, Src tyrosine kinase [39], H-Ras and K-Ras [40], the heme oxygenase [41]. Caveolae have also been associated with *bona fide* mechanosignaling pathways including MAP kinase, Akt, Src kinases, Rho and Rac small GTPases [42].

The central role of the caveolin scaffolding domain

A first study identified a domain responsible for the direct regulation of heterotrimeric G proteins by Cav1 [43]. This interaction was further confirmed for H-Ras, the Src and Fyn tyrosine kinases, and the endothelial nitric oxide synthase eNOS [35,40,41].

This specific feature of Cav1 resides in a particular α -helical domain (residues 82–101) called the caveolin scaffolding domain (CSD) so named because it is also required for Cav1 oligomerization. The CSD is localized on the N-terminal part but its relative position to the plasma membrane remains unclear. The CSD would exert an inhibitory role on signaling effectors by binding directly to a putative corresponding caveolin binding motif (CBM) identified in several of these effectors [35,40,41,44,49]. Indeed, biochemical studies on eNOS revealed the critical role of the CSD phenylalanine residue 92, which, by extending its lateral chain, allows to

Figure 2



Visualization of the caveolar coat at the plasma membrane of myotubes.

Survey view of the cytoplasmic surface of an unroofed mouse myotube presenting caveolae at the plasma membrane. Different types of caveolae structures are apparent, ranging from flat (1), circular (2), to fully budded (3). Scale bar: 500 nm. Scale bar in insets: 50 nm.

reach the hydrophobic pocket of eNOS to inhibit its catalytic activity [44,45]. A similar example was provided by the heme oxygenase 1 whose activity is inhibited through direct binding between its CBM domain and the CSD [41]. Furthermore, a small CSD-mimicking peptide inhibits eNOS activity whereas the corresponding mutated peptide increases eNOS activity most likely by competing with endogenous Cav1, which suggests a direct Cav1-CSD mediated inhibitory effect [46].

Controversy on CSD accessibility

However, new studies have recently undermined the model of signaling regulation through direct CBM/CSD interaction. The CBM motif was found to be poorly discriminative as it is also found in species such as *Saccharomyces cerevisiae* that do not express caveolins. No particular enrichment in proteins with the CBM sequence was found in the Cav1 interactome and the structural analysis of different CBM domains revealed that this domain was likely to be buried inside the proteins and therefore not readily available for interacting with the CSD [47,48]. Likewise, the CSD would also be an amphipathic helix and thus partially embedded inside the plasma membrane hence not accessible [49].

Alternative mechanisms could also be considered. Thus, a new study proposed that the CSD is a dynamic structure that can be either fully helical or partly unstructured, which may change its accessibility [50^{*}]. Furthermore, the conformation of Cav1 could vary with the oligomerization state and the organization of the caveola components (cavin isoforms or lipids). In this context, mechanical stress by promoting the release of Cav1 from disassembled caveolae [51] could not only control the ratio of caveolar vs. free Cav1 but also the accessibility to its CSD.

Yet the mechanisms controlling the reversibility of these interactions, a key parameter in signaling, remain to be explored. In this context, the post-translational modifications of Cav1 may be particularly relevant. Phosphorylation on Cav1 Ser 80 would result in a more versatile topology exposing the CSD because of charge repulsion between the inner leaflet of the plasma membrane and the phosphorylated residue [52]. A similar mechanism was proposed for Cav1 Tyr 14 phosphorylation that would form a stable structure facilitating binding to the CSD [53]. Cav1 Tyr 14 phosphorylation was also shown to prevent the direct binding of Cav1 on Egr1 [54]. Whether Cav1/Egr1 interaction occurs through the CSD is however unknown.

Indirect regulation of signaling by caveolae

Through caveolae

In addition to the direct inhibitory effect of Cav1 on signaling effectors, caveolae can also indirectly modulate intracellular signaling. Caveolae interact with the actin

cytoskeleton and contribute to lipid sorting and delivery to the plasma membrane through their enrichment in glycosphingolipids and cholesterol [55]. Caveolae can thus modulate the nanoscale plasma membrane organization, a key parameter in transmembrane receptor activation [56]. Once again this process could also be actively controlled by caveolae dynamics in response to mechanical strains [51,57]. In this regard, stretch-induced caveolae disassembly led to the redistribution of Cav1 and sphingolipids at the plasma membrane together with c-Src activation [58]. Furthermore, Cav1 depletion led to perturbations in Ras spatial nano-organization and signaling through changes in lipid composition and PS distribution [59^{*}]. In agreement with the localization of calcium pumps in caveolae [60], the mechanical disassembly of caveolae led to reduced Ca^{2+} responses through changes in $G\alpha_q$ /Cav1 association [58,61]. Finally, as for clathrin-mediated endocytosis, caveolae endocytosis, while limited under resting conditions, might also contribute to the endosomal control of signaling by delivering activated receptors to this compartment [62].

Through Cav2

The role of Cav2, which is not required for caveolae formation, remains enigmatic. Cav2 expression is required for activating the estrogen receptor α by 17 β -oestradiol [63]. The insulin signaling pathway can be regulated by Cav2 fatty acylation and phosphorylation, two successive post-translational modifications that prevent the interaction of the SOCS3 phosphatase with the insulin receptor allowing the activation of IRS-1 (Insulin Receptor Substrate-1) and the nuclear translocation of activated STAT3 [64–66]. Cav2 was allegedly reported to mediate signaling from the plasma membrane to the nucleus, as phospho-ERK nuclear translocation induced by insulin relied on Cav2^{154SSV156} C-terminal sequence [67].

Through cavins

Finally, the recently identified cavins may also contribute to caveolae signaling. Cavin-1 being essential to caveolae assembly, it controls the number of functional caveolae and thereby the localization of activated receptors in these structures [68,69]. Cavin-3 mediates ERK activation by anchoring caveolae to the plasma membrane via myosin-1c [70] and regulates their dynamics [71^{*}]. Likewise CAVIN-1/-2/-3 KO mice display a certain degree of heterogeneity among endothelial caveolae depending on tissues and cavin-2 expression [72].

Caveolinopathies

Because of the multiple functions of caveolae and their impact on signaling, it is not unexpected that several pathologies have been associated with caveolae dysfunction. With the notable exception of neurons and lymphocytes, caveolae are ubiquitously found in most cells and are particularly enriched in adipocytes, endothelial and

muscle cells. Accordingly, mutations or deregulation of caveolae components expression have been associated with lipodystrophy, vascular dysfunction, musculopathies [73] or cancer [74]. It is certainly no coincidence that, all chronically experience mechanical forces in their environment [1,51,75,76]. Whether these diseases can be related to defects in caveolae mechanosensing and mechanotransduction remains however to be tested.

Lipodystrophy

In mice, Cav1 or cavin-1 deficiency leads to a similar lipodystrophic phenotype consisting in important loss of fat mass associated with hypertriglyceridemia [10,77]. A p.Glu38X stop codon CAV1 homozygous mutation resulting in caveolae loss was first identified in a patient suffering from Berardinelli-Seip congenital lipodystrophy [78] followed by the identification in lipodystrophic patients of a homozygous frame shift mutation (c.696_697insC) in the PTRF gene coding for cavin-1 [79]. Other human mutations are found in Cav1 and cavin-1, including heterozygous frame shift mutations in the CAV1 gene (88delC and p.I134fsdelA-X137 in Ref. [80], p.Phe160X in Ref. [81^{*}]) and a single deletion in PTRF gene (c.947delA in Ref. [82]), leading again to a similar phenotype. These studies and others emphasize the central role of caveolae in lipid metabolism. Lipodystrophy could be ascribed to the loss of caveolae-dependent lipid storage capacities by adipocytes as mice lacking Cav1 or cavin-1 remain lean under high fat diet [77,80,83]. Cav1 is found at the plasma membrane of adipocytes and at the surface of lipid droplets, the key lipid storage organelle [84]. Thus, the absence of Cav1 will affect both lipid uptake and associated caveolae-dependent signaling events together with lipid droplet composition [85,86]. Lipid uptake results also in rapid swelling of adipocytes, a mechanical stress that may require functional caveolae for efficient membrane mechanoprotection.

Vascular dysfunction

In addition to lipodystrophy, mice lacking Cav1 and cavin-1 experience vascular dysfunction [87–89]. Defects in stimulated contractility, myogenic tone and endothelium-dependent relaxation of arteries have been observed in CAV1 KO mice [87] that could be related to the control of NO and calcium signaling by Cav1 [90,91]. The phenotype of CAV2 KO mice, which still have caveolae, is surprisingly restricted to lung dysfunction through increased lung endothelial cell proliferation [92]. Again shear stress is intrinsically associated with endothelial cells in vessels and caveolae are likely to be involved in several shear-related functions [93].

Muscular dystrophies and cardiomyopathies

Mutations of muscle-specific Cav3, cavin-1 and cavin-4 have been associated with several forms of muscular dystrophies and cardiomyopathies [79,94]. The observed symptoms could be ascribed to several defects in muscle

physiology. A lack of functional caveolae could lead to defective formation of the excitation contraction coupling machinery and disorganization of the T-tubule network [94,95]. It could also result in lipid homeostasis and mechanosensitivity defects upon muscle contraction, which in turn would directly affect the response of the sarcolemmal membrane to mechanical strains. Moreover, non-mutually exclusive caveolae-dependent defects have also been reported for signaling pathways important for muscle physiology such as those mediated by calcium, Akt or MAP kinases [96–98], or in the expression or localization of key proteins involved in membrane integrity or repair such as dysferlin [99], and also in mechanoprotection [51,100^{*}].

Cancer

Several thousand studies have addressed the role of Cav1 in this broad-spectrum pathology that is cancer. Yet this role remains complex with studies describing Cav1 as a tumor suppressor and others as an oncogene. The nature of this role may vary with the type and stage of cancer [74] and is probably related to a deregulation of signaling pathways involved in tumor progression. The other components of caveolae have been less studied. In breast and prostate cancers [101,102], Cav2 expression was increased whereas cavins, with lower expression, were more likely to be tumor suppressors [68,103,104]. The role of caveolae in cancer should be reconsidered through their mechanosensing function as recent data have shed light on the key role of mechanical forces in tumor progression [105].

Other pathologies including pulmonary arterial hypertension [106], fibrosis [87] or atherosclerosis [107] have also been associated with caveolae deficiency or deregulated Cav1 expression. These pathologies can also be analyzed in the context of mechanical dysfunction [32].

Conclusion and unresolved questions

Obviously more than 60 years after the first description of caveolae, many fundamental questions remain unanswered. It is intellectually challenging to reconcile the diversity of their cellular functions with a unique organelle that is mostly stable at the plasma membrane. The absence or deregulation of this ubiquitous organelle results likewise in a rather specific set of pathologies. A major unresolved question concerns the mechanisms by which caveolae can control so many signaling circuits. The recent structural description of the caveolar coat has been a major step forward that will allow to better understand how signaling effectors can associate with the different components of this compact structure. The resolution of the ternary structure of Cav1, as recently achieved for Cav3 [108], will bring new answers as well.

One could also speculate that the different Cav1 and cavin isoforms may assemble subpopulations of caveolae differing in their composition/structure thereby allowing customized signaling and local reactivity to mechanosensing in different cells and tissues. At the ultrastructural level, various degrees of caveolar invaginations can be observed (Figure 2). Whether these structures co-exist independently or are in dynamic exchange is unknown. It will be important to analyze the distribution of caveolin and cavin isoforms in these structures. Live cell imaging of caveolae with higher spatiotemporal resolution approaches such as super-resolution microscopy should also provide a better understanding of their significance [109**].

The rapid exchange of cavins and Cav1 from caveolae in response to mechanical constrains is potentially a new mechanism by which caveolae could control signaling in a highly dynamic and integrated manner [51,55,57,100,110*,111,112]. In this context, it is interesting that in most caveolae-associated pathologies, cells and tissues are subjected to increasing mechanical stress that may induce aberrant cellular mechanosignaling [105,113]. Thus, the miscellaneous functions of caveolae could be reunified through a central mechanoprotective role relying on mechanosensing and mechanosignaling. Thanks to the new conceptual and technological advances applied to caveolae biology during the past decade, we start to understand how this multifunctional organelle is capable of integrating and translating an array of various external stimuli into the regulation of distinct cellular functions. The next challenge is to translate this knowledge *in vivo* in order to design tailored therapeutics for treating caveolae-associated diseases.

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Titre : Rôle de la cavéoline-3 et de la mécanique des cavéoles dans la pathophysiologie du muscle.

Mots clés : Cavéoles, cavéoline-3, stress mécanique, dystrophie musculaire

Résumé : Les cavéoles sont des invaginations de la membrane plasmique qui nécessitent les cavéolines pour leur biogénèse. Récemment, mon laboratoire d'accueil a décrit un nouveau rôle pour les cavéoles dans la réponse au stress mécanique (Sinha et al, Cell, 2011). Des mutations de la cavéoline-3 (Cav3), isoforme spécifique du muscle, qui ont pour conséquence la rétention de la protéine dans l'appareil de Golgi, ont été décrites dans certaines dystrophies musculaires. Mon projet consiste en l'identification du lien fonctionnel entre les mutations de la cavéoline-3 et les dystrophies musculaires, qui ont comme phénotype principal un défaut d'intégrité et de réparation membranaire et des dérégulations dans l'homéostasie du muscle. Dans des myotubes humains provenant d'un patient portant la mutation Cav3-P28L ou Cav3-R26Q, j'ai pu montrer une diminution de la quantité de cavéoles à la membrane plasmique.

En conséquence, les myotubes mutants ne sont plus capables de tamponner l'augmentation de la tension membranaire provoquée par un stress mécanique, ce qui conduit à un défaut d'intégrité membranaire. J'ai aussi montré que la voie de l'interleukin-6 (IL6), importante pour l'homéostasie du muscle, est hyperactivée dans les myotubes mutants, révélant un rôle de régulateur négatif de la voie IL6 par Cav3. De plus, cette voie n'est plus régulée négativement quand un stress mécanique est appliqué comme c'est le cas dans les myotubes sauvages (WT). De manière intéressante, les myotubes mutés phénotypent une déplétion de Cav3 et ce phénotype est réversible lorsque l'on reforme des cavéoles à la membrane plasmiques des myotubes mutés en exprimant la forme WT de Cav3. Ceci confirme un lien direct entre les mutations de CAV3, l'absence de cavéoles et le phénotype observé.

Title : Role of caveolin-3 and caveolae mechanics in the muscle pathophysiology.

Keywords : caveolae, caveolin-3, mechanical stress, muscular dystrophy

Abstract : Caveolae are plasma membrane invaginations that require caveolin proteins for their biogenesis. Recently, our laboratory reported a new role for caveolae in the cell response to mechanical stress (Sinha et al, Cell, 2011). Mutations in the CAV3 gene (muscle isoform), which lead to caveolin-3 (Cav3) retention in the Golgi apparatus, are associated with muscular dystrophies. My project consists in identifying the functional link between Cav3 mutations and MDs, which exhibit defects in membrane integrity and repair, and in muscle homeostasis. In Cav3 P28L and Cav3 R26Q mutated human myotubes, I showed a lack of caveolae structures at the plasma membrane. This results in a lack of membrane tension

buffering upon mechanical stress, leading to mechanoprotection defects. I also showed that the interleukin-6 (IL6) pathway, important for muscle homeostasis, is overactivated in mutant myotubes, showing evidence of a negative regulation of the pathway by Cav3. Furthermore, the IL6 pathway is no longer negatively regulated upon mechanical stress, as it is the case in wild-type (WT) myotubes. Interestingly, mutated myotubes behave as Cav3 depleted myotubes, and the phenotype is reversible with caveolae reformation upon expression of the WT form of Cav3. This confirms the direct link between CAV3 mutations, the absence of caveolae and the observed phenotype.

