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Intercellular Communication in Skeletal Muscle Stem Cell Niche: Focus on extracellular vesicles and secreted signals

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LIST OF PAPERS

The work developed during this PhD *Thesis* collects the following scientific manuscripts under preparation for submission in international peer-reviewed journals:

Romancino DP*, **Buffa V***, Ferrara I, Raccosta S, Notaro A, Caruso S, Noto R, Martorana V, Cupane A, Giallongo A, d'Azzo A, Manno M, Bongiovanni A. *Palmitoylation is a post-translational modification of Alix regulating the membrane organization of exosomes*. *equal contribution. ***Manuscript in preparation*** [*Thesis Chapter I*]

Buffa V, Ollitrault D, Marazzi G, Sassoon D. *Regulation of satellite cells self-renewal mediated by fibro-adipogenic progenitors*. ***Manuscript ongoing***. [*Thesis Chapter II*]

Buffa V, Bongiovanni A and Romancino DP. *An improved and straightforward Acyl Biotin Exchange (ABE) method to detect protein S-palmitoylation using PVDF membranes.* ***Manuscript in preparation***.

Ollitrault D, **Buffa V**, Correra R, Hoareau B, Pöhle-Jronawitter S, Stricker S, Marazzi G, Sassoon D. *Glucose-deprivation induces interstitial progenitor vasculogenesis.* ***Submitted.***

ABSTRACT

Cell-to-cell communication is an essential requirement within the muscle stem cell niche to ensure processes as development, homeostasis and regeneration. The communication among the different cellular components of the niche can occur through direct contact or can be indirect, when mediated by soluble factors or extracellular vesicles (EVs).

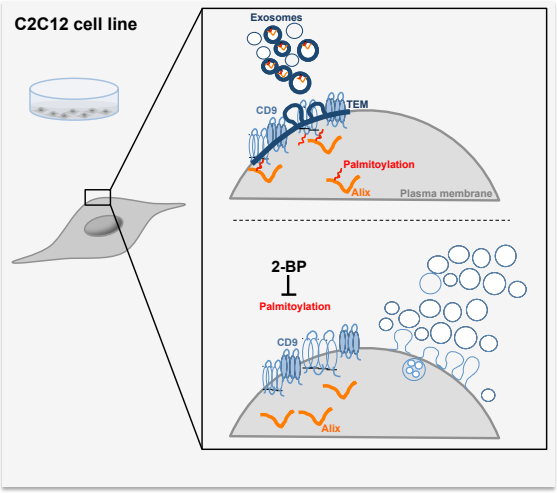
Myogenic cells release Alix positive EVs through budding from the plasma membrane. Here we applied biophysical and biochemical methods to investigate the mechanisms underlying Alix positive extracellular vesicles biogenesis and cargo loading. We determined that protein palmitoylation is important for EV production, modulating the interaction between EV specific regulators (i.e. Alix and CD9). Moreover, we observed that the inhibition of this post-translational modification altered the structural organization of the EV lipid bilayer.

In the muscle stem cell niche, the indirect communication can also be mediated by paracrine signals derived from tissue-resident mesenchymal stromal cells, called as fibro-adipogenic progenitors (FAPs). Importantly, we found that FAPs conditioned media induced the growth of satellite cells in 3D clusters with aciniform morphology and enriched in Pax7 positive and MyoD negative cells. This cell signature is typically observed in quiescent satellite cells of non-injured muscle, suggesting that FAPs conditioned media in vitro promoted satellite cells self-renewal. In addition, our preliminary results suggest that a member of the bone morphogenetic proteins (BMPs) group of growth factors is released by FAPs, representing the potential trigger for satellite cell self-renewal. Together, these data bring new insights into the active participation of FAPs in the muscle niche and their regulation of satellite cells fate.

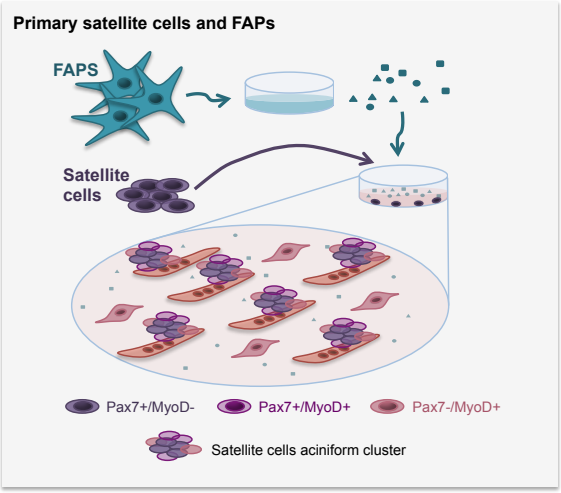
Unraveling the mechanisms regulating muscle stem cell niche communication, either via soluble factors or EVs, is crucial to better understand the fundamental processes involved in muscle homeostasis and regeneration.

GRAPHYCAL ABSTRACT

Indirect Communication
Exosomes



Direct/Indirect Communications
Satellite cells – Myotubes – FAPs



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LIST OF NON-STANDARD ABBREVIATIONS

2BP	2-bromopalmitate
AA	amino acid
AFM	Atomic force microscopy
AIP1	ALG2 interacting protein
ALG-2	Apoptosis-linked-gene 2
APT	Acylprotein thioesterase enzyme
ARRD1	Arrestin Domain Containing 1
BAX	BCL2-associated X protein
cAMP	Cyclic adenosine monophosphate
CEP55	Centrosomal protein 55
CHMP4	Charged multivesicular body protein 4
Cys	cysteine
DLS	Dynamic light scattering
DRM	detergent-resistant membrane
DTT	Dithiothreitol
DTX	Diphtheria toxin
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal Sorting Complex Required for Transport
EVs	Extracellular vesicles
FACS	Fluorescence-activated cell sorting
FAPS	Fibro-adipogenic progenitors
FGF2	Fibroblast growth factor-2

GEM	Glycolipid-enriched membrane microdomains
GO	Gene ontology
GPI	glycophosphatidylinositol
HAM	Hydroxylamine
HC	High-confident
HD-PTP	His domain phosphotyrosine phosphatase
HDAC	Histone de-acetylase
HGF	Hepatocyte growth factor
HIV 1	Human immunodeficiency virus type 1
HRP	horseradish peroxidase
IDE	Insulin-degrading enzyme
IGFs	Insulin-like growth factors
IL	Interleukin
ILVs	Intraluminal vesicles
IP-ABE	Immunoprecipitation-Acyl-Biotin Exchange
LAMPs	Lysosomal-associated membrane protein
LBPA	Lysobisphosphatidic acid
LIF	Leukemia Inhibitory Factor
MHC	Major histocompatibility complex
MSCs	Mesenchymal stem cells
MT1-MMP	Membrane-type 1 matrix metalloproteinase
MVBs	Multivesicular bodies
NEM	N-Ethylmaleimide
PARP	Poly (ADP-ribose) polymerase
PAT	Protein acyl-transferase enzyme
PDCD6IP	Programmed cell death 6 interacting protein
PDGF-AA	Platelet-derived growth factor-AA

PRR	Proline-rich region
PTMs	Post-translation modifications
PVDF	Polyvinildifluoride
SANS	Small angle neutron scattering
SAXS	Small angle X-ray scattering
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SETA	SH3-encoding expressed in tumorigenic astrocytes
SkM	Skeletal muscle
TEM	Tetraspanin-enriched microdomains
TNTs	Tunneling nanotubes
TSG101	Tumor susceptibility gene 101
VEGF	Vascular endothelial growth factor
BMPs	Bone morphogenetic proteins
TGF	Transforming growth factor
AM	Amplification medium
DMEM	Dulbecco’s modified eagle medium
bFGF	Basic fibroblast growth factor

INTRODUCTION

1 Intercellular communication

Cell-cell communication is an essential requirement of multicellular organisms that drives development, maintenance and survival as a whole body; the impairment of cell signaling and communication leads to disease (Alberts B, 2002). Cell-cell communication occurs by different signaling mechanisms such as: i) direct communication between proximal cells and ii) indirect communication that occurs between cells that are distant. Cell communication comprises three stages: signal reception from the initiating cell, signal transduction via intracellular pathways, and the response from the receiving cell.

1.1 Direct communication

Direct communication involves a permanent or a transient physical contact when cells are very close to each other, and molecules of the cell membrane interact by chemical (e.g. cytokines, hormones or neurotransmitters) or mechanical stimuli (e.g. contraction force and electrical). Direct communication can be subdivided based on the molecules responsible for the signaling pathway: tight junctions, anchoring junctions, gap junctions and membrane receptors (Juliano, 2002). Amongst the several proteins involved in this tight communication, connexin 43 composes the gap junction (Ishido and Kasuga, 2015). Gap junctions are composed of two juxtaposed hemi-channels present on the surface membrane of two adjacent cells. When in contact, the hemi-channels form a trans-cellular channel allowing small molecules, as ions and metabolites to propagate rapidly between adjacent cells. Adherent junctions, composed by cadherins, have been reported to regulate not only cell-cell adherence but also stem cell interaction within the niche, being relevant for their maintenance, proliferation and migration (Marthiens et al., 2010). More recently, another type of satellite cell-myofiber communication was described, namely tunneling nanotubes (TNTs). TNTs are ultrafine membrane structures (Rustom et al., 2004), these structures participate in the satellite cells direct communication with other satellite cells, their progeny and muscle fibers, allowing the direct passage of cytosolic components as nucleic acids, mitochondria and calcium ions (Tavi et al., 2010).

1.2 Indirect communication

Indirect communication occurs without physical contact between the cells that are communicating, and is characterized by the extracellular release (secretion) of messages, which ultimately will arrive to the responder cells. The signaling mediated by secreted factors is classified based on the distance between communicating cells and target cells: autocrine - when messages are directed to the same cell which produced the signal; paracrine - to cells in close proximity; and endocrine - if cells communicating are distant and the message is only reachable through the blood flow. Several types of messengers have been identified and categorized as: small soluble factors (i.e. growth factors, cytokines, lipids or acid nucleic) or cell-delivered extracellular membranous vesicles (EVs).

1.2.1 INDIRECT SIGNALING MEDIATED BY SECRETED FACTORS IN SKELETAL MUSCLE

Skeletal muscle is an important regulator of whole body metabolism, and as the biggest organ of the body, all secreted muscle-derived signaling metabolites will reach other tissues. One of the most well studied biomolecules in skeletal muscle is the lactic acid and its role in Cori cycle. Lactic acid is produced by muscle and can be either transformed to lactate to be consumed by muscle to produce energy (paracrine effect), or act locally when muscle is hypoxic, allowing to enhance the oxygen intake, which is an important factor in the oxidation process of lactate cycle (autocrine effect) (Ibrahim et al., 2017). Following intense exercise, the blood lactate levels signal the liver in order to break-down the stored glycogen and to deliver systemic glucose that assists energy production in the hypoxic skeletal muscle tissue (Chang et al., 2015). At the same time, high lactate levels in blood are also indicators of low levels of acetyl-CoA (an important player in oxidative respiration) to adipose tissue in order to inhibit the fatty acid release because its consumption is highly dependent upon the levels of this factor (Boyd et al., 1974); and to lung, where the high levels of lactate in blood under hypoxic conditions increase ventilation (Chang et al., 2015). Overall, lactic acid/lactate is mostly produced by muscle in situations of exercise, producing an effect at different levels of the body (autocrine, paracrine and endocrine) (Ibrahim et al., 2017). Other bioactive molecules, such as adenosine, cAMP, b-aminoisobutyric acid and 3-hydroxyisobutyrate have been also shown to play important roles in muscle communication with other tissue to promote homeostasis of the body (Baar et al., 2002) (Chiavegatti et al., 2008).

Skeletal muscle is also a key endocrine organ because of its ability to release muscle-specific cytokines, referred to as myokines, in response to contraction. Myokines mediate muscle communication locally via autocrine-paracrine signaling in muscle tissue, but also with distant organs, such as adipose tissue and liver, participating in metabolic homeostasis (Figure 1) (Pedersen and Febbraio, 2008). Myokine IL-6 was described by Steensberg et al. (Steensberg et al., 2000), and due to its secretion during muscle contraction it was named an “exercise factor” (Pedersen and Febbraio, 2008). Apart from IL-6, several other myokines have been described; some examples are listed in the table 1.

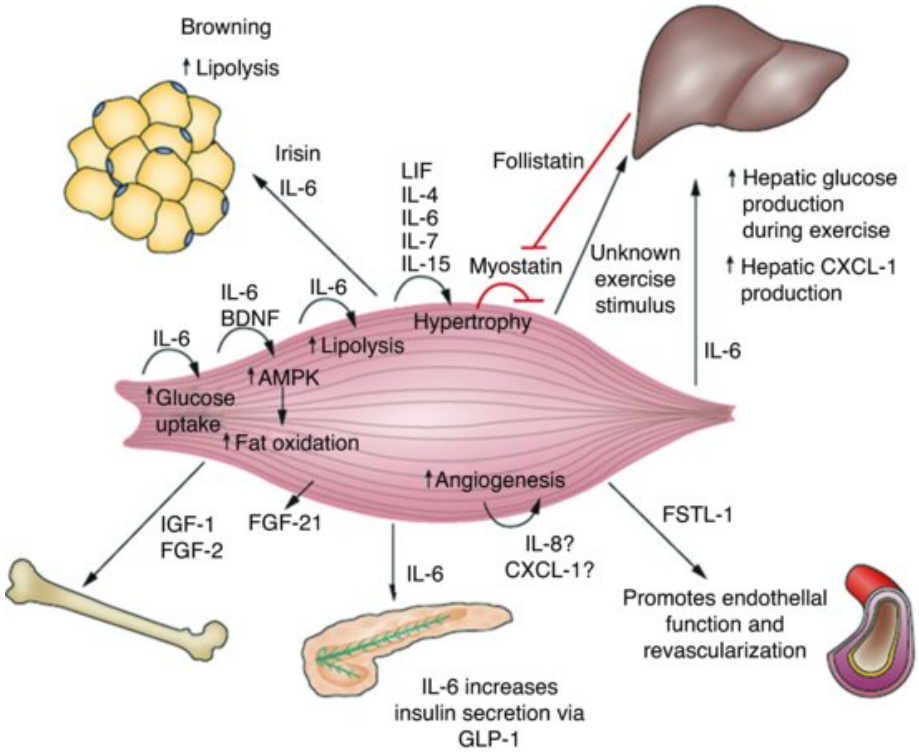

Figure 1. Muscle as a secretory organ. Skeletal muscle releases a plethora of molecules acting both on muscle cells as paracrine/autocrine factors and on different organs as endocrine signals. For example, leukemia inhibitory factor (LIF), interleukin (IL)-4, IL-6, IL-7, and IL-15 promote muscle hypertrophy, that is inhibited in turn by myostatin. IL-6 locally enhances insulin-stimulated glucose uptake, while it has systemic effects on the liver and adipose tissue.
Adapted from (Pedersen, 2013).

Table 1. List of main myokines and their function

Myokine	Function	Reference
IL-6	As endocrine factors, it influences metabolism, increasing adipolysis and hepatic glucose production, and as an anti-inflammatory signal it promotes glucose uptake, increases lipolysis and fatty acid oxidation. As paracrine factor, it stimulates proliferation of muscle stem cells.	(Steensberg et al., 2000) (Pedersen and Febbraio, 2008) (Kim et al., 2004) (Steensberg et al., 2003) (Serrano et al., 2008)
LIF (Leukemia Inhibitory Factor)	It is related to hypertrophy and regeneration (induction of satellite cells proliferation and suppression of inflammatory response).	(Kurek et al., 1997) (Broholm et al., 2011) (Hunt et al., 2013)
IL-15	It is expressed by muscle fibers with anabolic effects on skeletal muscle, and probably it acts in the cross talk between muscle and fat.	(Furmanczyk and Quinn, 2003) (Argiles et al., 2005)
IL-7	It is involved in the migration of satellite cells, but not in the proliferation of these cells.	(Haugen et al., 2010)
Myostatin	Potent inhibitor of skeletal muscle growth. It inhibits proliferation and differentiation of satellite cells. As endocrine factor, affect metabolism and growth of different tissues, as fat and liver.	(Allen et al., 2011)

2 Extracellular vesicles – Exosomes

More recently, an evolutionary conserved mechanism of communication has been identified with the identification of extracellular vesicles (EVs). EVs are membrane-enclosed vesicles released by cells for information transfer. Proteins, lipids or genetic material can be packaged in EVs and transmitted to local or distal target cells. Several EV subtypes have been defined based upon their specific functions, size or biogenesis (Gould and Raposo, 2013). EVs can be isolated by different techniques, the most widely used is described in detail by Thery et al, (Thery et al., 2006) in which the size of the vesicles isolated depends on the speed of centrifugation; smaller vesicles (exosomes) are obtained by larger speeds of centrifugation. Other methods have been developed including sucrose gradients, and polymer-based precipitation or immunocapture by antibody-coated beads (applied in some commercial kits). None of these methodologies are ideal for the isolation of a perfectly pure population of EVs, considering the high heterogeneity of the EVs (Colombo et al., 2013). Accordingly, two main classes of EVs have been defined: the exosomes – nano-size vesicles (below 150nm in diameter) originating from the endosome compartment, where they are formed by inward budding of the membrane in multivesicular bodies (MVBs) to be released in the extracellular milieu after as MVB fusion with the plasma membrane (Figure 2) (Cocucci and Meldolesi, 2015); and the ectosomes - also named as microvesicles, nanoparticles, membrane particles, originating directly from the plasma membrane budding typically as a bigger diameter EVs (100-1000nm) (Figure 2) (Stein and Luzio, 1991). It is also included in this class of vesicles apoptotic bodies and large oncosomes, deriving from membrane blebbing of apoptotic and cancer cells, respectively (Ciardiello et al., 2016).

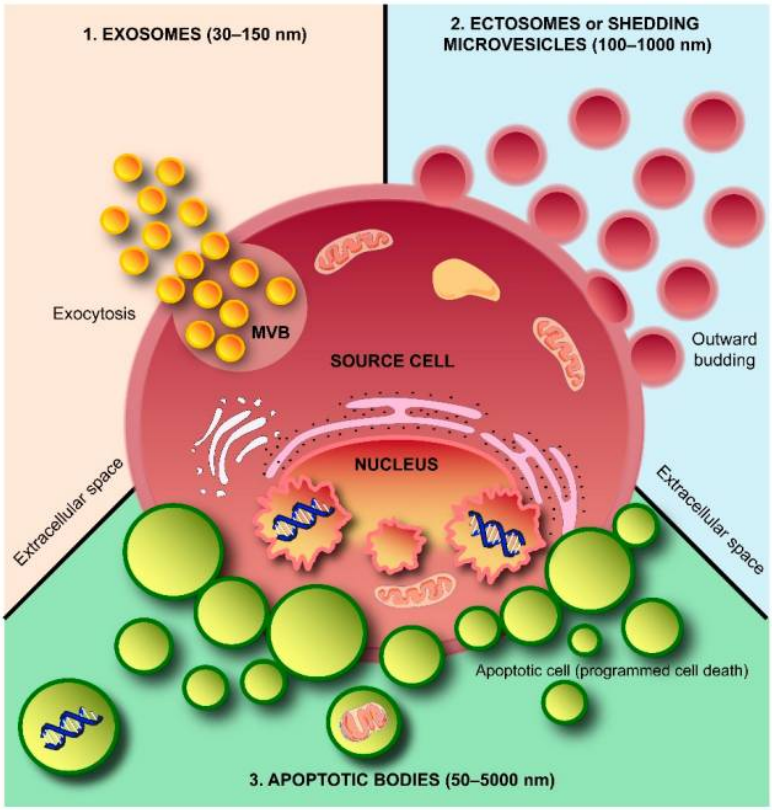

Figure 2. A model of different types of extracellular vesicles formed by cells during physiological processes. Exosomes, ectosomes and apoptotic bodies.
Adapted from (Kalra et al., 2016).

2.1 Extracellular vesicles biogenesis

EVs can originate from almost any cell type. The EV cargo (vesicle content) is defined during EV generation, which represents then an important focus of EVs to use them as potential clinical tool for the development of new therapies. Although the nomenclature is still a matter of debate (Gould and Raposo, 2013), the EV biogenesis has also been used to categorize the different subtypes of EVs (exosomes and ectosomes).

Exosomes formation has been traditionally described to be dependent upon the endocytic pathway, and can be divided into three different phases: i) internalization of trans-membrane proteins and cellular elements, in the early endosome ii) inward budding at the limiting endosomal membrane, forming the intraluminal vesicles (ILVs) II); and iii) the secretion of exosomes (Figure 4) (Thery et al., 2002). Exosomes originate during the internal definition of the cargo within the ILVs, forming the intermediate organelle called multivesicular bodies (MVBs). The future of exosomes is

highly dependent upon the cycle of the MVBs, which can either fuse with lysosomes undertaking the degradative lysosomal pathway, or fuse with the plasma membrane, releasing exosomes in the extracellular space (Figure 4) (Kowal et al., 2014).

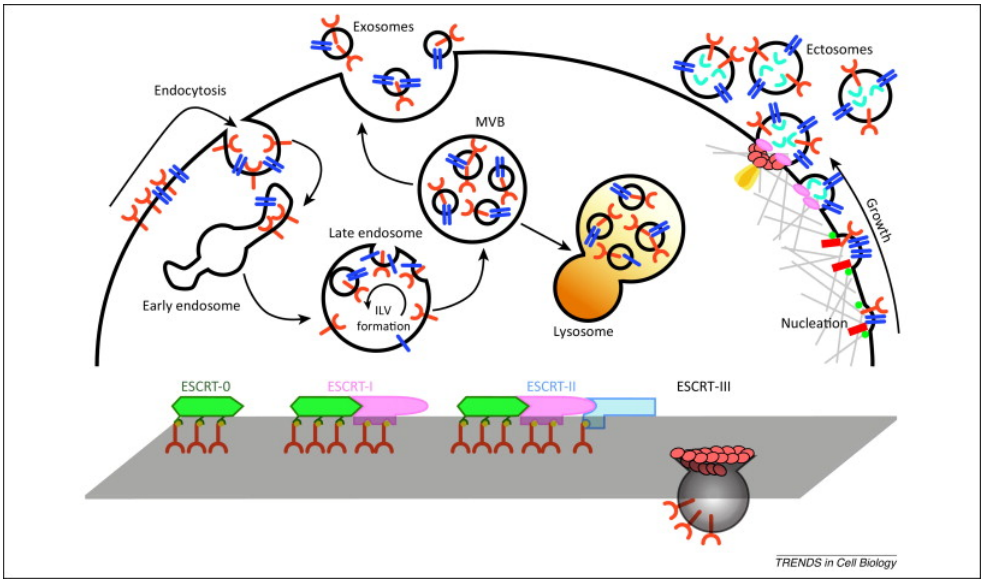

Figure 3. Extracellular vesicles biogeneisis. Exosomes are formed in the endocityc pathway, as inward budding of the late endosome, giving rise to the MVB containing the ILVs. ILVs are released as exosomes after MVB fusion with plasma-membrane. Ectosomes bud directly from the plasma membrane.
Adapted from (Cocucci and Meldolesi, 2015).

It was proposed (Colombo et al., 2014) (Stuffers et al., 2009) (Trajkovic et al., 2008) that the inward budding of the endosomal membrane, to form the ILVs and the internal accumulation of the cargo are essential steps in exosome formation regulated by multiple protein complexes (Figure 5). It was suggested that the Endosomal Sorting Complex Required for Transport (ESCRT) machinery is essential for the accumulation and processing of ubiquitinated proteins in the budding ILVs (Wollert and Hurley, 2010). The ESCRT is composed by four sub-complexes each with a specific role. ESCRT-0 binds and directs the ubiquitinated proteins towards endosomal membrane; ESCRT- I and ESCRT-II start the local budding; and ESCRT-III interacts with the enzyme HD-PTP, cooperates in protein de-ubiquitination and also scission of ILVs (Colombo et al., 2013) (Cocucci and Meldolesi, 2015). The ESCRT complex also interacts with other proteins, such as Alix, to collect the luminal cargo (Hurley and Odorizzi, 2012).

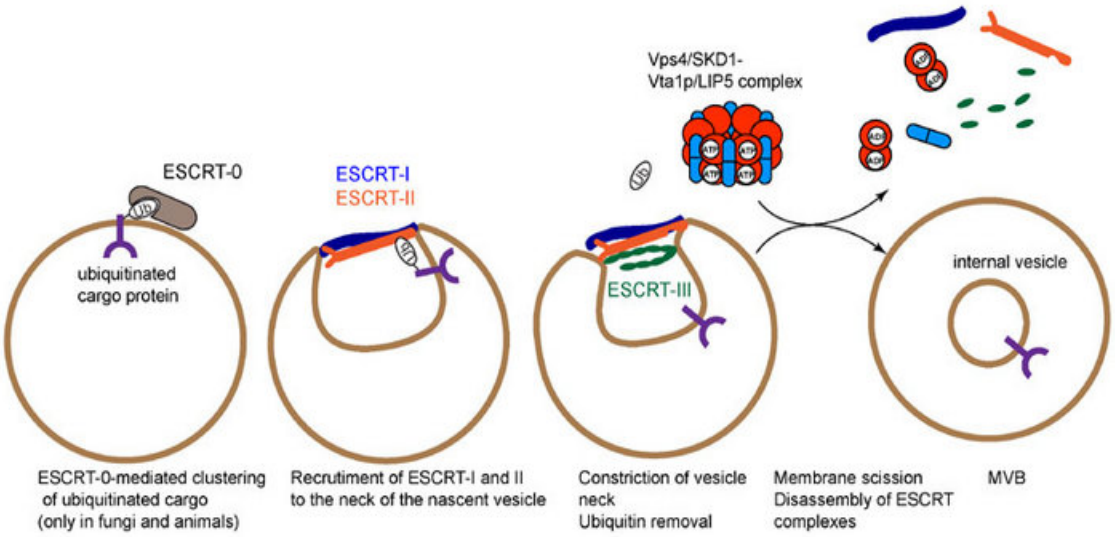

Figure 4. Scheme of ESCRT complexes involvement during formation of intraluminal vesicles.
Adapted from (Wollert and Hurley, 2010).

In addition to the ESCRT, other complexes can participate in the regulation of the initial steps exosomes biogenesis, like complexes dependent on lipids/ceramide or tetraspanin dependent (Figure 6) (Colombo et al., 2014) (Stuffers et al., 2009) (Trajkovic et al., 2008). However, it is not yet completely unravel whether those complexes act together or not in the same MVBs, and/or if they concern different exosomes.

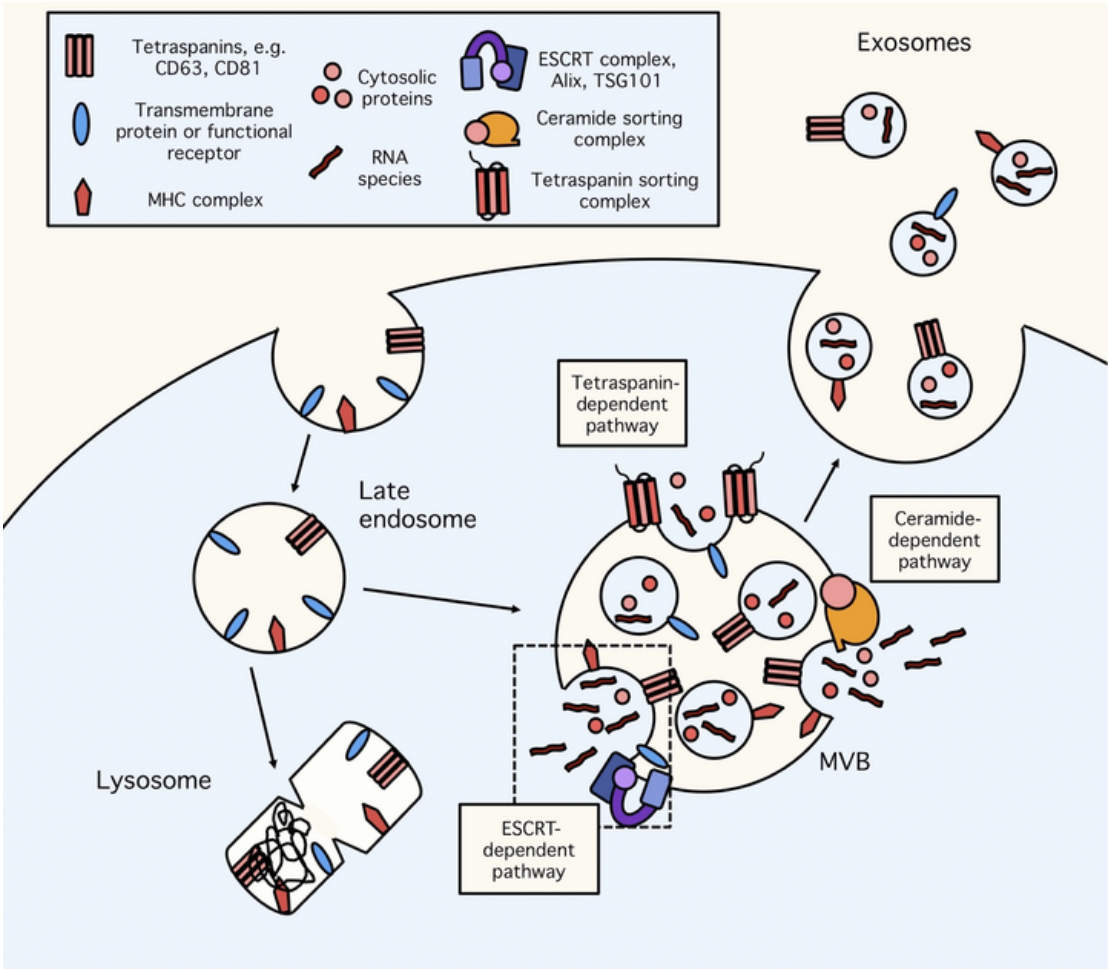

Figure 5. Molecular complexes for exosome biogenesis. Multiple pathways are engaged in biogenesis of intraluminal vesicles of multivesicular bodies (MVBs) and consequently of exosomes: Endosomal sorting complex required for transport (ESCRT) components, lipids, and tetraspanins.
Adapted from (Miller and Grunewald, 2015).

The second main class of EVs, usually named ectosomes, is formed by outward budding directly from the plasma membrane. The direct budding process starts with the nucleation of the plasma membrane, clustering of membrane constituents and their rearrangement (cholesterol, sphingomyelin and its product ceramide), the cytoskeleton disintegration and the recruitment of protein for the membrane abscission (Kalra et al., 2016) (Cocucci et al., 2009) (Bianco et al., 2009). In addition, it has been shown that the ESCRT complex, or at least some subunits, are important for the accumulation of the cargo protein in the lumen of the ectosomes (Van Engelenburg et al., 2014).

The formation of EVs has been described by two independent processes (the endosomal compartment-dependent budding or the direct budding direct from membrane), but it is not clear if these two pathways are completely independent or

overlap. It has been shown that exosome-like vesicles can originate by plasma membrane direct budding (Booth et al., 2006) (Romancino et al., 2013) thus the classification of exosomes and ectosomes is not certain. In this regard, Booth and colleagues (Booth et al., 2006) divided exosome biogenesis in two alternative modes: delayed and immediate. Delayed mode is the traditional exosome biogenesis process, dependent on the endosomal compartment, and thus more steps are involved (mediated by the ILVs formation in the late endosomes followed by their released). Conversely, the immediate mode was recently described involving the direct budding of exosomes from the plasma membrane, in particular from endosome like-domains (Gould and Raposo, 2013). These highly dynamic microdomains contain a lipid composition typically present in MVB membrane, they are enriched in MVBs proteins as CD63, CD81, and the budding requires some components of the ESCRT complexes. Taking in consideration all these features, authors considered these vesicles as exosomes and re-defined the exosomes origin as being not only dependent on MVBs origin, but also direct plasma membrane budding (Gould et al., 2003) (Booth et al., 2006).

Another important point is the fact that the isolation/selection of the vesicles is typically performed using high-speed centrifugation and thus this selection is completely dependent on the size of the particles and not on their origin. Following this, it cannot be excluded that some of the exosome that have been studied contain a mixture of vesicles with both endosomal and plasma membrane origin (Thery et al., 2002). Furthermore, it has been suggested that micro and nanoparticles are also generated after the sampling and during the harvesting procedure as a result of cell fragmentation due to shear forces; these membranous particles could contribute to the EVs in the isolates (Sustar et al., 2011) (Stukelj et al., 2017).

Considering the difficulties in unambiguously defining the origin of vesicles, it is accepted the use of exosomes term in a less strict manner Considering the difficulties in unambiguously define the origin of vesicles, it is accepted the use of exosomes term in a less strict manner (Kowal et al., 2014). In this thesis, I will refer to microvesicles to the population of larger vesicles that are isolated by the 10000xg centrifugation methodology and to exosomes or exosome-like vesicles the population isolated with successive 118000 x g ultracentrifugation technique.

2.2 Extracellular vesicles biochemical composition

EVs are cell-derived membranous structures with a specific protein pattern (Figure 3). Some of the membrane proteins are found in secreted vesicles whereas others are in general present in the majority of exosomes regardless of cell type origin. These *bona fide* exosome proteins derive from the endosomal compartment and cytosol or plasma membrane, such as tetraspanins CD63, CD9, CD81, the major histocompatibility complex (MHC) I and II, lysosomal-associated membrane protein (LAMPs), ras superfamily of monomeric G proteins (Rab, G-protein) RAB, flotillin-1, adhesion molecules and integrins. In contrast, nuclear, mitochondrial, endoplasmic reticulum or Golgi complex proteins are absent in exosomes (Colombo et al., 2014) (Thery et al., 2002). The EV is enriched in specialized membrane components which are functional lipid clusters and proteins, including tetraspanin-enriched microdomains, sphingomyelin, phosphatidylserine and cholesterol, as well in ganglioside GM3 and ceramide. The presence of sphingomyelin and cholesterol and their associated proteins (flotilins or GPI-anchored proteins) is a typical feature of the lipid rafts subdomains (Tan et al., 2013). In addition, the EV surface membrane shows several enzymes with proteolytic (membrane-type 1 matrix metalloproteinase (MT1-MMP) and insulin-degrading enzyme (IDE)) and glycolytic (sialidase and heparanase) activities. Since these surface enzymes remain active, their presence in the EVs can be responsible for growth factors mobilization or degradation in extracellular matrix macromolecules (Sanderson et al., 2017).

The EV lumen is also rich in biomolecules, where proteins from cytoskeleton and ESCRT (Endosomal Sorting Complex Required for Transport) proteins, (Alix, TSG101, Syntenin, VSP4), as well as nucleic acids (mRNAs, siRNAs, and long noncoding RNA) can be found (Colombo et al., 2014).

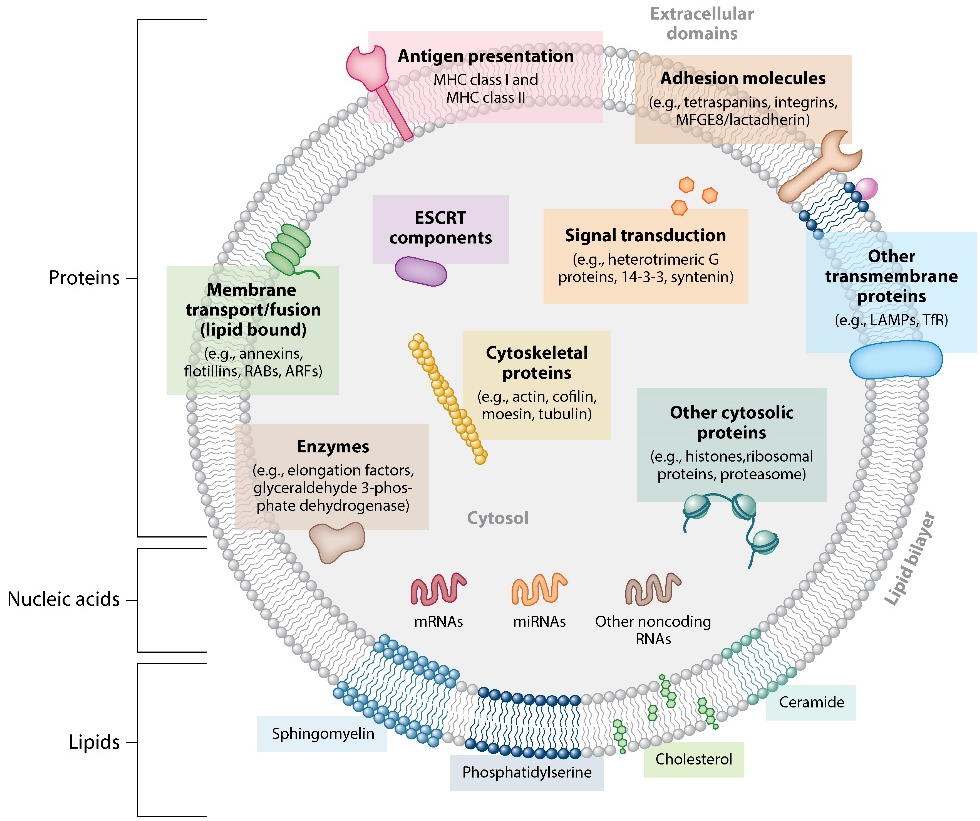

Figure 6. Biochemical features of extracellular vesicles.
Adapted from (Colombo et al., 2014).

2.2.1 *PROTEIN POST-TRANSLATION MODIFICATIONS IN EV REGULATION –*

2.1 S-PALMITOYLATION

Post-translation modifications (PTMs) consist in the addition of molecules of diverse nature (nucleotides, amino acids, carbohydrates, lipids or other chemical groups) on specific amino acids of a given protein, by specialized enzymes. PTMs allow proteins to upgrade their structure during or after protein biogenesis, and thus to change their stability, interaction sites, and cellular location. This process increases the protein diversity, enabling their fine regulation in the biological processes. There is a growing interest in the role of PTMs of proteins that signal EV biogenesis, being ubiquitination, phosphorylation and oxidation some of the investigated PTMs (reviewed in (Moreno-Gonzalo et al., 2017).

Proteomics studies found EVs produced by myeloid-derived suppressor cells enriched in ubiquitinated endosomal trafficking proteins (Burke et al., 2014). Ubiquitinated proteins, such as ARRD1 have been shown to be anchored to the arrestin domain of

the plasma membrane, allowing direct membrane budding (Nabhan et al., 2012). SUMOylation is another example of PTMs involved in EV regulation and α-synuclein (lipid membrane-associated protein) incorporation in exosomes through ESCRT machinery (Kunadt et al., 2015). In the case of phosphorylation, it was demonstrated that the phosphorylated form of aquaporin 2 protein has been identified in urinary EVs (Gonzales et al., 2009), and this could reveal an increase in vasopressin (Fenton et al., 2008). Glycosylation has been shown to be important both in the EV formation and in their uptake. In 2012, Baietti et al. (Baietti et al., 2012), demonstrated the interaction between syndecans (a heparan sulphate proteoglycan), syntenin and Alix in the process of ESCRT-dependent exosome biogenesis. These proteins are essential for EV uptake and the hydrolysis of the glycol group blocks this process (Escrevente et al., 2011).

Lipidations are also important PTMs of membrane proteins that determine their function. Different membrane anchors or lipid-anchored proteins, such as prenylated proteins, fatty acylated proteins (N-myristoylation or S-palmitoylation) and glycosylphosphatidylinositol-linked proteins (GPI), allow the targeting of highly oligomeric cytoplasmic proteins into secreted vesicles that bud directly form the plasma membrane (Shen et al., 2011) (Yang and Gould, 2013). Recently it has been reported another palmitoylation and isoprenylation motif present at the C-terminus of human RhoB protein, this promotes intraluminal vesicles delivery of proteins, and therefore in exosomes by the endosomal pathway (Oeste et al., 2014).

S-palmitoylation consists in the addition of a saturated fatty acid chain, palmitic acid (16:0) to sulfhydryl group on the cysteine residue of proteins via thioester linkage (Fukata and Fukata, 2010). This lipidation is regulated by acyl-transferase (PAT) and acylprotein thioesterase (APT) enzymes, which can add or remove the thioester bond, respectively. Contrary to other lipid PTMs (i.e. N-mirystoilation or prenylation), S-palmitoylation is a reversible modification, conferring to the protein to which the palmitoyl group is associated more dynamism in the interaction with the plasma membrane, thus playing a role in the signaling processes. S-palmitoylation can also regulate protein subcellular localization, stability and trafficking (Aicart-Ramos et al., 2011).

2.2.2 SURFACE MEMBRANE SPECIALIZED DOMAINS – TETRASPANIN ENRICHED MICRODOMAINS

Highly ordered plasma membrane regions, such as lipid raft, tetraspanin-enriched microdomains (TEM) and glycolipid-enriched membrane microdomains (GEM), have

been implicated in exosome biogenesis. Tetraspanins are small proteins with four transmembrane domains and are largely represented in the majority of the cells. CD9, CD81, D63 are just some example of molecules belonging to this superfamily, and often associated with EVs. These proteins have the ability to cluster together as well as with other transmembrane proteins forming small and specific membrane domains, TEMs (Hemler, 2005) (Yanez-Mo et al., 2009).

Different studies have highlighted the potential role of TEMs in EV biogenesis, demonstrating the key role of tetraspanins in ESCRT-independent pathway of exosomes formation. For instance, EV secretion is diminished when CD9 is knockout (Chairoungdua et al., 2010), while when CD81 is depleted, EV composition is altered (Perez-Hernandez et al., 2013). TEMs may also play a role in the physical organization of EVs, regulating vesicular budding and membrane-curved structures (Wrigley et al., 2000). Shen and colleagues demonstrated that targeting proteins to EVs demands the plasma membrane binding and the higher-order oligomerization, features that tetraspanins fulfill (Shen et al., 2011) (Fang et al., 2007). To ensure the proper EV cargo, different plasma membrane anchors, and among the others the palmitoylation tag, can serve as link for cytoplasmatic proteins to the route towards plasma membrane/EVs (Shen et al., 2011). S-palmitoylation is the most common PTM found in tetraspanins, being important in their interactions and organization in the TEMs (Charrin et al., 2002). Taking together, palmitoylation drives crucial steps in membrane trafficking and EV formation, it is possible that controlling interactions between EV regulators and tetraspanins provides a fine tuning of EV biogenesis involving the TEMs.

2.2.3 CYTOSOLIC COMPONENTS – ALIX

Alix (also known as programmed cell death 6 interacting protein (PDCD6IP) or AIP1) is a cytosolic protein, first identified for its pro-apoptotic function and calcium-dependent interaction with the apoptosis-linked-gene 2 (ALG-2). Alix interacts with different other proteins as an adaptor involved in a plethora of cellular mechanisms (Figure 7 and Table 2), including endocytic membrane trafficking and cytoskeletal remodeling.

Table 2. Biological functions of Alix.

Biological processes	Description	References
Apoptosis	Alix acts as partner of ALG-2, which is implicate in both extrinsic and intrinsic apoptosis. Numerous	(Chen and Sytkowski, 2005)

	results suggested the link between Alix, ALG-2 and apoptosis, and the overexpression of Alix was associated with in vivo cell death and caspase cascade activation.	(Rao et al., 2004) (Blum et al., 2004) (Mahul-Mellier et al., 2006)
Endosomal trafficking and exosome biogenesis	Alix has a key role in the protein sorting during the formation of the ILVs in the MVB. Different ESCRT complexes cooperate to allow the loading on the ubiquitinated proteins during ILVs formation. Alix has been shown to interact with CHMP4 subunit of the ESCRT- III complex, functioning as a crucial adaptor for protein sorting in ILVs, and thus also in exosomes. Moreover, recently it has been reported the Alix regulates not only the cargo loading but also triggers vesicles formation. It is the case in the mechanism of exosomes biogenesis based on syndecan/syntenin: the sorting of the transmembrane receptor syndecan is depending on the cytosolic recruitment of syntenin, Alix binds syntenin, ensure the link with ESCRT III and the vesicle budding. In addition, Alix it has been shown to interact with lysobisphosphatidic acid (LBPA), a phospholipid specific of late endosome, and with the ESCRT II via the subunit TSG101.	(Bissig and Gruenberg, 2014) (Baietti et al., 2012) (Bissig et al., 2013) (Romancino et al., 2013)
Viral budding	It has been demonstrated that Alix bind a motif (YPLTSL), present in the protein Gag of HIV 1 (human immunodeficiency virus type 1). This domain, interacting with Alix and with other endosomal complexes, allows the virions to bud from the plasma membrane.	(Martin-Serrano et al., 2003) (Strack et al., 2003)
Cytokinesis	Carlon et al. 2008 have shown that Alix is enrolled to midbody of cells that are dividing, and it interacts with Cep55. The Cep55/Alix/ESCRT-III interactions are, in turn, crucial in the late phases of cytokinesis.	(Carlton et al., 2008)
Growth factor	Alix exerts a negative role in regulation of EGFR	(Schmidt et al., 2004)

receptor endocytosis	endocytosis. It inhibits the interaction of the SETA- endophilin complex with the E3 ubiquitin ligase Cbl, and in this way decreases the Cbl-mediated ubiquitylation of EGFR, leading to impairment in EGFR downregulation.	
Filopodia formation and cell migration	Alix has been reported to contribute in cytoskeleton remodeling interacting with F-actin, α-actinin, cortactin, and focal adhesion kinase. In skeletal muscle cell, Bongiovanni et al showed actually that Alix silencing leads to alteration in cell morphology, compromised formation of sarcolemmal protrusions, and consequently impaired cell motility.	(Pan et al., 2006) (Bongiovanni et al., 2012)

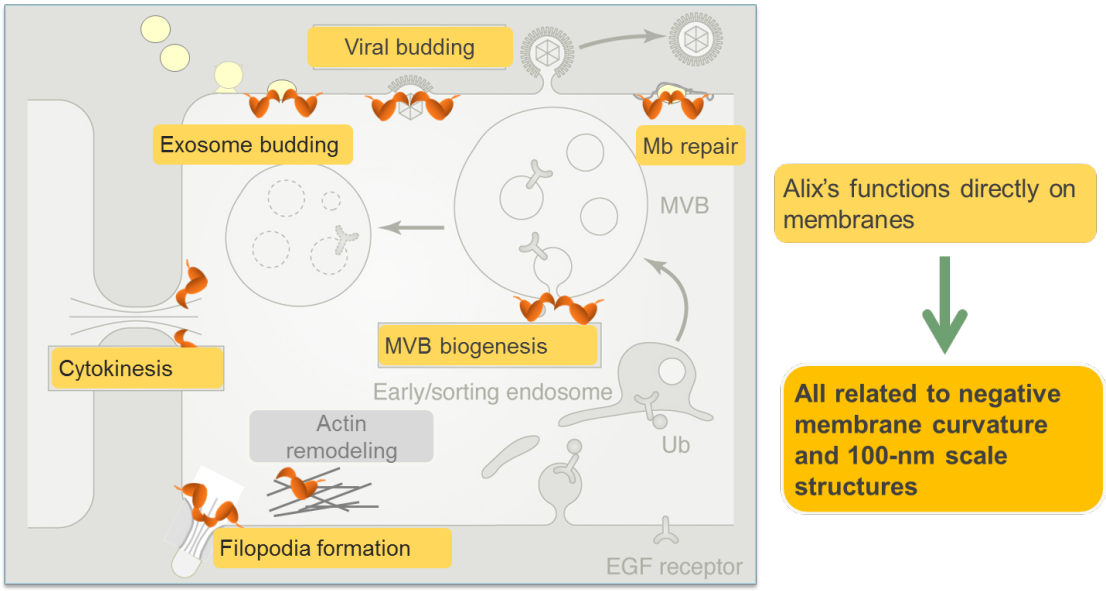

Figure 7. Multiple functions of Alix in cellular processes.
Adapted from (Odorizzi, 2006)

These multiple abilities of Alix to act in different cellular processes are due to its complex architecture with different domains, which include: a N- terminal Bro1 domain (residues 1–358), a central "V" domain (residues 362–702) and a C-terminal proline-rich region (PRR, residues 703–868) (Figure 8a) (Fisher et al., 2007). The Bro1 domain has a banana shape, and is responsible for the endosomal localization of Alix, binding the ESCRT III subunit CHMP4 (Zhai et al., 2011a). Moreover, the convex face of Bro1 domain is enriched in high electropositive residues, a feature that favors the interaction with the negatively charged membrane. Therefore, it is possible that Alix is related in the formation of the negative curvature, a common feature among the membrane remodeling process in which it is involved (Kim et al., 2005) (Bongiovanni et al., 2012). The V-domain is constituted by two extended three-helix bundles and binds to the LYPXL motif. This motif has been shown to regulate cargo loading in exosomes with the interaction between the Alix V-domain and syntenin (Baietti et al., 2012). The C-terminal proline-rich region (PRR) is the most common site of interaction in Alix, and it is predicted to lack a persistent secondary or tertiary structure (Fisher et al., 2007). The PRR region is determinate to define the Alix conformation as described in Zhai model (Figure 8b): when the C-terminal (PRR region) is associated with Bro-V domain, the protein is in a closed and auto-inhibitory state, while the release of this association allows the opening of the V domain, allowing Alix dimerization (Zhai et al., 2011b).

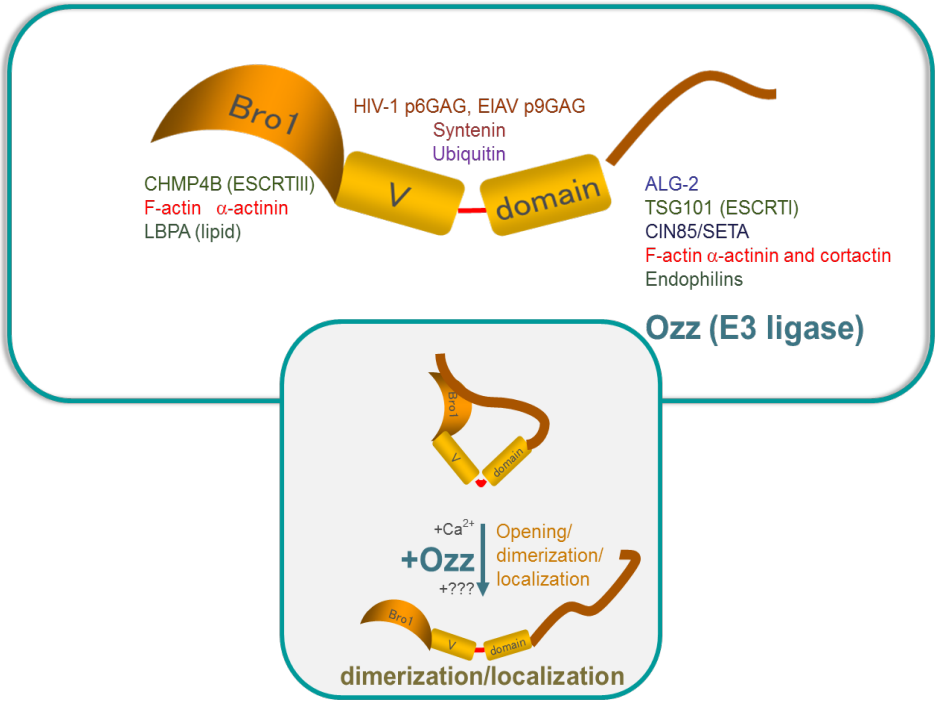

Figure 8. Alix's protein domains and the model of the two conformations of Alix; the "closed" conformation of Alix, where the PRR interacts with the Bro1 domain, and the "open" conformation, prone to dimerization.

Recently, our group has reported that Alix characterizes the exosomes derived from skeletal muscle cells, which are formed from direct budding of plasma membrane. The release of this myoblast-derived exosomes is increased during muscle differentiation. Considering these observations, the peculiarity of the membrane remodeling and the dynamics of skeletal muscle cells, it has been proposed an immediate mode of exosomes release (Booth et al., 2006). Interestingly, Alix loss-of-function results have shown an altered release of exosomes from C2C12 myoblasts, revealing that Alix is not only a marker of these exosomes, but also plays a crucial role in their formation and regulation (Romancino et al., 2013).

2.3 Extracellular vesicle role in skeletal muscle

As EVs can be produced by all types of cells, they may have a broad action in biological processes using either their ligands to stimulate receptor activation/repression in the target cell, or delivering the cargo effectors inside the recipient cell (Andaloussi et al., 2013). It was already described the importance of EVs in tissue repair (Gatti et al., 2011), immune surveillance (Raposo et al., 1996), blood

coagulation (Del Conde et al., 2005) and stem cell maintenance (Ratajczak et al., 2006). At the same time, it has been studied the role of EVs in pathologic context, and one of the most analyzed field has been the oncobiology. EVs are known to contribute in the establishment of a pre-metastatic niche (Peinado et al., 2012), to promote tumor progression (Skog et al., 2008), proliferation, angiogenesis (Al-Nedawi et al., 2009), matrix remodeling (Sidhu et al., 2004) and immune-escape (Cai et al., 2012).

In the context of the skeletal muscle acting as an endocrine organ, there is increasing evidence that muscle signals both locally and in systemically. As for other tissues and systems, different studies have started to unravel the potential roles of EV secretion in muscle. The Guescini and Bongiovanni's labs identified EVs released from a skeletal muscle cell line (C2C12) and primary myoblasts that are positive for the exosomal marker Alix and TSG101 (Guescini et al., 2010) (Romancino et al., 2013). Secretome profiling of human myoblasts has revealed two different populations of EVs, likely regulated by different mechanisms and involved in diverse processes (Le Bihan et al., 2012).

EVs from myoblasts enclosed numerous signal molecules as myogenic growth factors linked to muscle development, as for example fibroblast growth factor-2 (FGF2), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), and platelet-derived growth factor-AA (PDGF-AA) (Choi et al., 2016). Considering in vivo studies, it has been suggested a role in the regeneration process for muscle derived EVs (Matsuzaka et al., 2016) (Choi et al., 2016). Nakamura and his group have also used an in vivo injury model, to show the regenerative potential of mesenchymal stem cells (MSCs) derived exosomes on skeletal muscle (Nakamura et al., 2015). Moreover, muscle derived EVs have been found in human bloodstream. These vesicles positive for alpha-sarcoglycan, are enriched for miR-206 (Guescini et al., 2015), a miRNA specifically expressed in muscle tissues. The same miRNA (miR206) was found in EVs derived from muscle stem cells progenitors, they were shown to be important to regulate the fibrogenic cell collagen expression, and therefore the extracellular matrix (ECM) remodeling during myofiber growth (Fry et al., 2017). Muscle derived EVs could then play a role, locally, in repair and regeneration processes, as well as they could have a long distance effect, as regulators of body homeostasis and representing also a helpful biomarker.

In the context of a disease, EVs can be enriched of specific pathological molecules, and for this reason be used to find "biomarkers" for early diagnosis or to monitor the prognosis. Since EVs can be found in several biological fluids, as for example blood and urine, biomarkers on EVs could allow the so called "liquid biopsy" (Boukouris and Mathivanan, 2015). Considering the involvement of EVs in various pathological

conditions, it is possible that a beneficial effect can be obtained by inhibiting the production or the release of EVs, or by blocking their uptake. The potential approaches are reviewed by Andaloussi et al (Andaloussi et al., 2013); for example decreasing the EV production, by inhibiting the ceramide formation, or the interactions syntenin–syndecan, or by blocking the tetraspanins. In addition, exosomes could be an excellent vehicle for drug delivery. Reflecting the membrane composition of donor cell, EVs being non-immunogenic, they can avoid the fast clearance from circulation and therefore efficiently arrive to the specific target tissues (Johnsen et al., 2014) (Wiklander et al., 2015). It is possible to load the therapeutic drug into the exosomes (as chemical drug, proteins or nucleic acids) with diverse approaches, acting on the producing cells or on the isolated EVs (Kim and Kim, 2017). Moreover, EVs have specific cell tropism (Lai et al., 2013) (Kooijmans et al., 2016) thanks to different adhesion molecules and surface ligands. Thus, EVs can be engineered to express a target specific ligand, using for example a plasmid for the ligand fused with an exosomal membrane protein (Alvarez-Erviti et al., 2011).

To this purpose, it is important to understand the mechanisms of protein loading into exosomes, as the role of PTMs of proteins that will be sorted on EVs. This could help in engineering therapeutic exosomes using a tag or a PTM to target the specific protein into exosomes, or as well to correlate a particular modified protein to specific pathological condition, serving as high specific markers in management of a disease.

3 Skeletal muscle niche

The muscle stem cell niche allows for the direct communication between the differentiated myotubes and muscle stem cells (i.e. satellite cells), which are located between the basement membrane and the sarcolemma of fibers. Direct contact between these two cell types contributes to the regulation of satellite cell quiescence during homeostasis and activation in response to injury.

3.1 Satellite cells self-renewal

Skeletal muscle constitutes the largest tissue of the body with the main function of skeletal movement (Gollnick and Matoba, 1984) and consists of multinucleate striated muscle myofibers, each surrounded by a basal lamina. Satellite cells were identified by Mauro in 1961 (Mauro, 1961) based upon their specific anatomical location and represent the resident muscle progenitors cells (Figure 9).

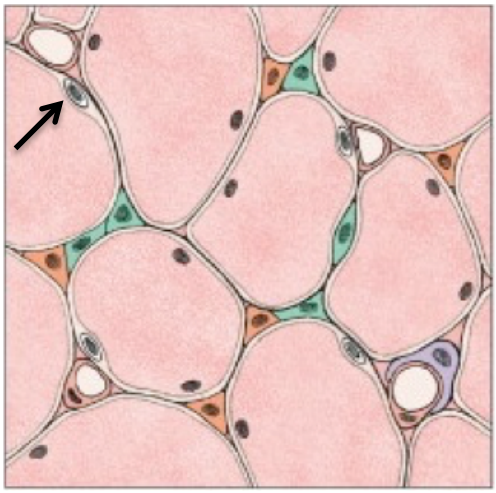

Figure 9. The muscle stem cell niche. A cross-section of the adult muscle, composed of myofibers, satellite cells (indicated by the arrow) reside beneath the basal lamina of the myofiber. In the interstitial space there are the mesenchymal progenitors (green), the connective tissue cells (orange), and the blood vessels, including endothelial cells (pink) and the associated pericytes (purple).
Adapted from (Pannerec et al., 2012)

In adult muscle, satellite cells are generally in a quiescent state and express Pax7, M-cadherin and α7-Integrin (Bismuth and Relaix, 2010) (Buckingham and Montarras, 2008) (Irintchev et al., 1994) (Yin et al., 2013). After injury, satellite cells re-enter in the

cell cycle and expand followed by differentiation to repair and replace damaged myofibers (Charge and Rudnicki, 2004). Pax7 has been described as a key factor for maintenance of the undifferentiated state of adult satellite cells (Gunther et al., 2013) (von Maltzahn et al., 2013). The myogenic differentiation program starts with the expression of Myf5 and MyoD followed by Pax7 down-regulation concomitant with the expression of terminal differentiation genes as myogenin (Myog) (Figure 10) (Bismuth and Relaix, 2010) (Sassoon et al., 1989).

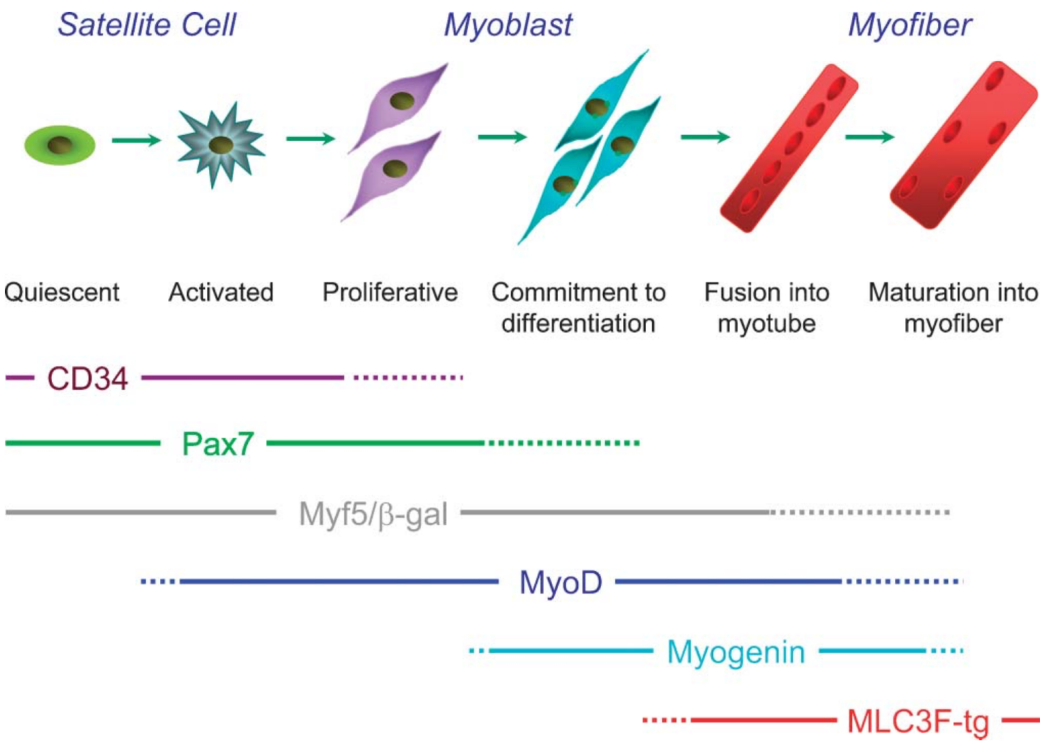

Figure 10. Scheme of satellite cell myogenesis and typical markers for each state.
Adapted from (Zammit et al., 2006)

Stem cells, by definition, have the ability to give rise to differentiated cells in their progeny as well as to replenish the stem cell pool (self-renewal). This capacity reflects the unique feature of stem cells to divide not only symmetrically, as the majority of somatic cells do, giving rise to two similar daughters cells; but also asymmetrically, resulting in two different daughters cells, one with stem cell properties and another committed to differentiation. In the case of satellite cells, different signals derived from the basal lamina and the myofiber determine the type of segregation of the DNA

strands and protein factors and ultimately will determine the final cell fate of the daughter cells. Thus, the division parallel to the fiber will lead to symmetrical division of satellite cells and replenish of the satellite cell pool, while an apical-basal division (perpendicular to the myofiber) will lead to an asymmetric outcome and the differentiation of the one of the daughter cells (Bentzinger et al., 2012). By use of lineage tracing models, it has been demonstrated that Pax7+/Myf5- satellite cells, through apical-basal division, give rise to a basal Pax7+/Myf5- cell (satellite cell) and an apical Pax7+/Myf5+ cell (muscle-committed cell) (Kuang et al., 2007). It is possible that the satellite cell population is a heterogeneous population constituted by stem cells and committed progenitors, thus two models have been proposed to explain satellite cell self-renewal and differentiation (Figure 11): i) the satellite cell reservoir is composed both by Pax7+/Myf5- satellite cells and Pax7+/Myf5+ cells and that cells expressing only Pax7 undergo symmetric or asymmetric division to amplify or replenish, respectively, while the committed cells (Pax7+Myf5+) mainly undergo symmetric division to a terminal differentiation state; ii) in response to injury, quiescent (Pax7+) satellite cell are activated and proliferative (Pax7+/MyoD+). The majority of the cells resulting from proliferation differentiate while some re-enter a Pax7+/Myf5- state and renew the quiescent stem cell pool (Relaix and Marcelle, 2009).

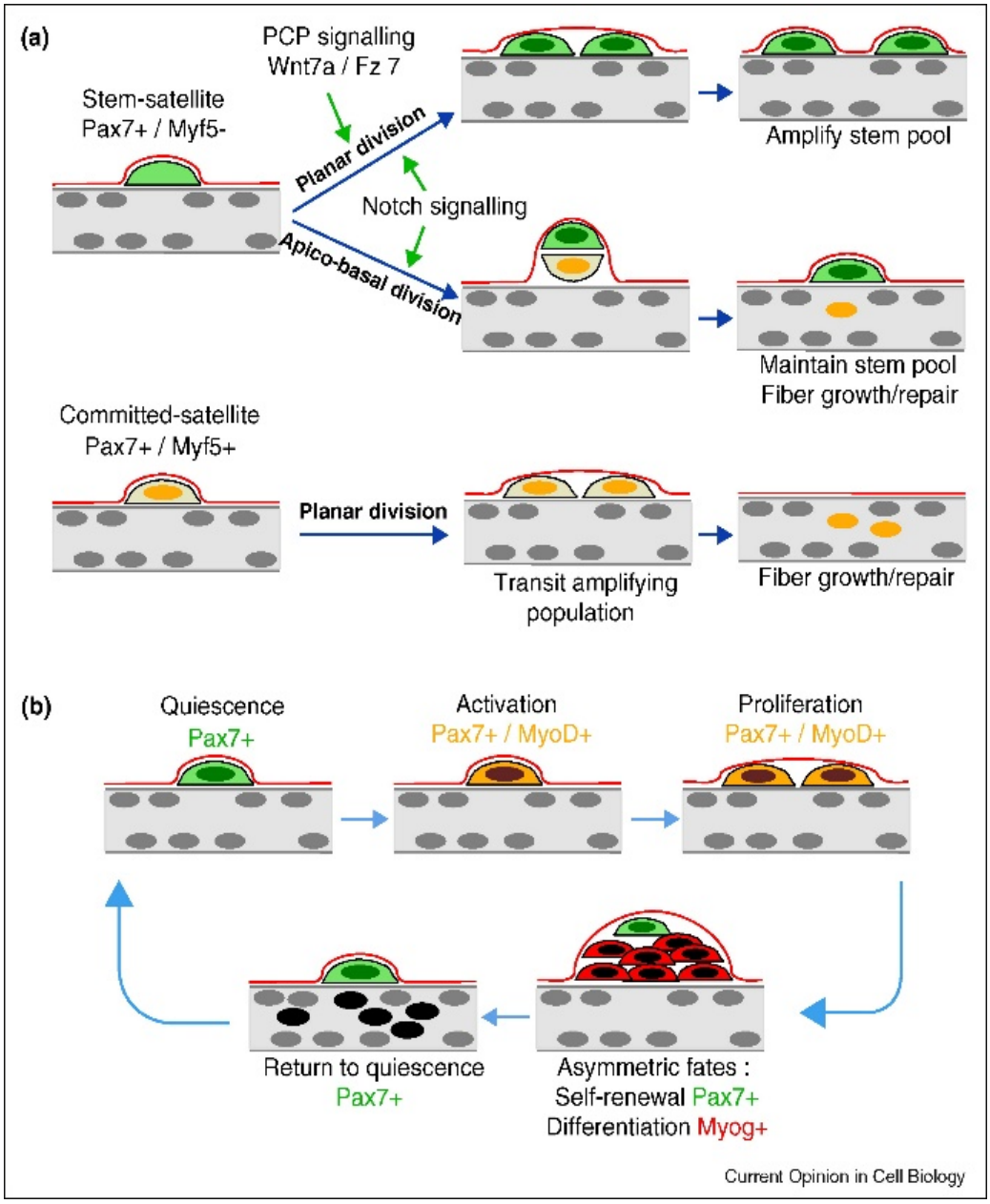

Figure 11. The two proposed models to describe the self-renewal and differentiation of satellite cells in muscles.
Adapted from (Relaix and Marcelle, 2009)

It is known that the tissue microenvironment has an impact in the balance between the maintenance of the reversible quiescent state of stem cells and their ability to re-enter in proliferation. In the steady-state condition, the satellite cells occupy a distinct position in muscle and any perturbation of the niche, either physiological or pathological, such as discontinuity of the myofibers or ECM (direct contact), paracrine signals, oxygen and nutrients availability, and mechanical stress impact their performance. In line with this notion, many procedures used to study satellite cell

biology require removing them from their niche and thus may impact their behavior (Rojas-Rios and Gonzalez-Reyes, 2014).

3.2 Muscle stem cell niche and the regeneration process

The stem cell niche constitutes the specific microenvironment responsible for maintaining stem cell competence. In the case of satellite cells in homeostatic conditions, the main niche components are the muscle fibers. It has been reported that satellite cell activation results from disrupting contact between the satellite cells and the myofiber sarcolemma (Bischoff, 1986). Moreover, Notch receptors have been described as crucial to maintain satellite cells quiescence (Bjornson et al., 2012), and since Notch receptors are activated with ligands presented by juxtaposed cells, it is likely that Notch signals are most likely to be presented by the myofibers (Bentzinger et al., 2013) (Koch et al., 2013). M-cadherin and the glycoprotein CD34 are required for the adhesion of satellite cells to myofibers (Irintchev et al., 1994) (Beauchamp et al., 2000). In particular, it has been reported that N-cadherin and M-cadherin, expressed by satellite cells, participating to the direct link with the myofiber act as important regulator of quiescence, and their removal lead to satellite cell activation (Goel et al., 2017). Satellite cells also directly interact with the fiber basal membrane through the integrins α7 and β1 and dystroglycan (Blanco-Bose et al., 2001) (Cohn et al., 2002). Additionally, heparan sulphate proteoglycans Sdc-3 and Sdc-4, present on satellite cell surface, act as co-receptors for integrins and help to better interact with the ECM and soluble factors (Xian et al., 2010).

Although satellite cells and muscle fibers are essential players in the stem cell niche and in the regeneration process, there are other important cellular components in the muscle tissue formation. After injury, the first response arrives from immune cells (Elhelu, 1983). Infiltrating monocytes, leukocytes and activated macrophages act in the clearance of cell debris formed after muscle tissue death. Following the clearing process, macrophages secrete anti-inflammatory factors in order to confine the inflammatory response (Bosurgi et al., 2012). It is important to highlight, that macrophages exert also a role on myogenic cells, inducing their proliferation during the pro-inflammatory phase and their differentiation along with the anti-inflammatory wave. These effects are mediated by paracrine signals as well as by deposition of ECM proteins (Gratchev et al., 2001). As described in Figure 12, after the inflammatory response, fibro-adipogenic progenitors (FAPs), fibroblasts and vascular cells

(endothelial and smooth muscle cells) start their job in the de novo formation of the muscle tissue (Bentzinger et al., 2013). When muscle is damaged, together with the muscle fibers also the microvasculature system is destroyed, leading to a hypoxic state. New vasculature structures are then required, thus the number of capillaries increases after injury, and only after four weeks their levels go back as in steady state (Luque et al., 1995). Endothelial cells secrete different signals to satellite cells such as IGF-1, FGF, HGF and VEGF (Christov et al., 2007). These promote satellite cell proliferation, and in turn differentiating myoblasts enhance angiogenesis. In addition, a role for vascular smooth muscle cells in maintenance of quiescent satellite cells has been shown, as they negatively regulate satellite cell proliferation and differentiation by secretion of Angiopoietin-1 (Abou-Khalil et al., 2009). When muscle regeneration occurs to re-establish the architecture disrupted in the stem cell niche, a dynamic reorganization of the extracellular environment occurs (Goetsch et al., 2003). The fibrogenic mesenchymal stromal cells, such as fibro-adipogenic progenitors (FAPs) and fibroblasts are involved in the remodeling of the niche, since they release the fibrillar ECM (Tomasek et al., 2002).

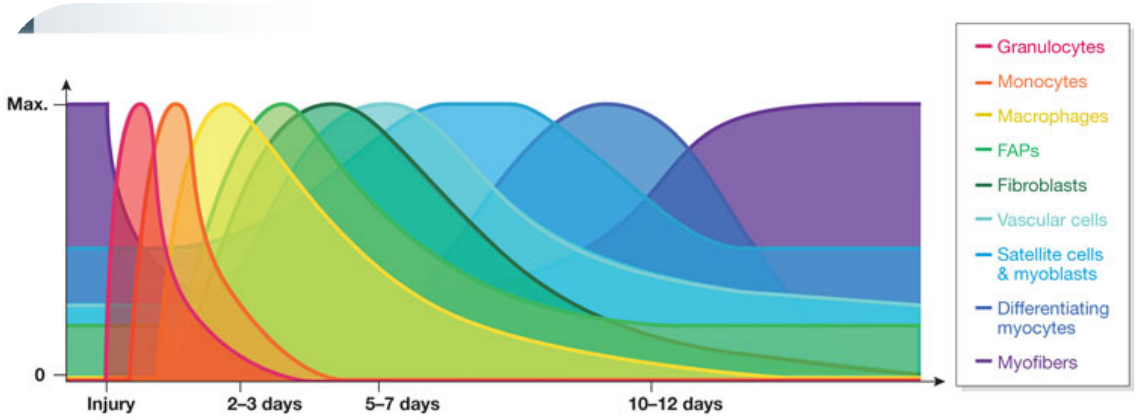

Figure 12. Participation of the different cell types in muscle regeneration.
The relative presence of immune, fibrotic, vascular and myogenic cell types after muscle injury. Adapted from (Bentzinger et al., 2013).

Moreover, it has been reported that genetically ablation of connective tissue fibroblast resulted in the premature differentiation of satellite cells and defective muscle regeneration (Murphy et al., 2011). In the muscle stem cell niche has been described also a population of resident non-satellite cells (PW1-positive interstitial cells) with myogenic potential (Mitchell et al., 2010). The role of these cells is anyway still unclear, considering that the genetic ablation of the satellite cells is sufficient to abolish the regenerative capacity. When satellite cells are depleted, after injury also the normal

fibroblast response is impaired (Murphy et al., 2011). This highlights the complex interconnected feature of the stem cell niche, where probably satellite cells do not represent only the stem cell pool, but an active actor needed to orchestrate the other cells. In the light of the function of all players in the muscle regeneration, it is becoming clear that both the direct and indirect signals are essential for the balance between satellite cells self-renewal and differentiation in muscle fibers.

3.2.1 FIBRO-ADIPOGENIC PROGENITORS CROSS TALK WITH SATELLITE CELLS ON THE MUSCLE FIBER

FAPs are a skeletal muscle resident population, capable to differentiate into fibroblast and adipocytes, when regeneration fails, but also to promote a successful regeneration by communicating with the muscle stem cells niche (paracrine signals) and by producing the essential ECM after the inflammatory cells clearance (Uezumi et al., 2010) (Joe et al., 2010) (Natarajan et al., 2010). Because of their multiple roles in muscle regeneration and repair processes, FAPs have been defined as “a double-edged sword in skeletal muscle regeneration” (Natarajan et al., 2010). According to the model proposed by Natarajan and colleagues and shown in Figure 13, in response to injury FAPs are activated, start to proliferate and stimulate satellite cells to differentiation. When the newly differentiated myoblasts and myofibers are formed, they inhibit FAPs differentiation and survival, which leads to FAP cells death. This negative feed-back mechanism is important to control the excessive expansion FAPs and to restore the muscle homeostasis. When regeneration of new myofibers fails, the balanced signaling between the stem niche components is lost, and FAPs, lacking of the inhibitory signals, repair the damage muscle with fibrotic and fatty tissue (Natarajan et al., 2010). Successful direct and indirect reciprocal communications between FAPs and satellite cells, as well as among them and the rest of the muscle niche (i.e. muscle myofibers) are critical to proper regeneration of damaged muscle. FAPs are found in the interstitial space between myofibers, near blood vessels, but outside the basal lamina (Joe et al., 2010). FAPs activity starts to be regulated early in the regeneration process by the inflammatory cells: it has been reported that eosinophils determine FAPs fate through IL-4, and the inactivation of this cytokine leads FAPs to form adipocytes (Heredia et al., 2013). FAPs differentiation is also regulated by the muscle myofiber, as was shown in the co-culture system of FAPs and regenerating myofibers, resulting in a significant inhibition of FAPs adipogenesis (Uezumi et al., 2010). Secreted factors (insulin-like growth factor 1 (IGF-1), hepatocyte growth factor (HGF) and nitric oxide) released by endothelial cells and macrophages also regulate FAPs

activity (Heredia et al., 2013) (Cordani et al., 2014) (Tatsumi et al., 2002) (Takada et al., 2012)

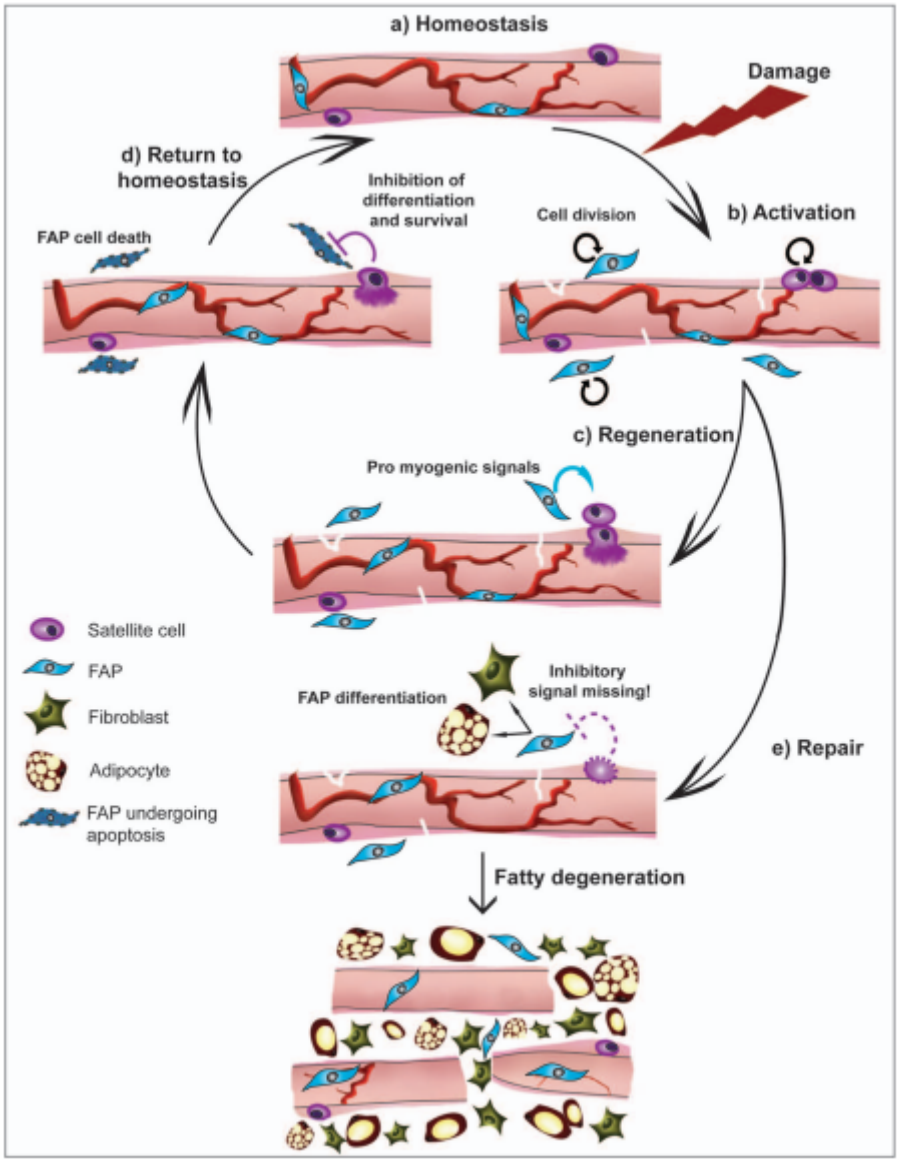

Figure 13. Model of FAPs involvement in regeneration and repair. Adapted from (Natarajan et al., 2010).

FAPs can directly promote myogenic differentiation of satellite cells (Joe et al., 2010) (Mathew et al., 2011) even though the molecular mechanism involved in this process has not yet been clearly unraveled. Mozzetta and colleagues reported that FAPs isolated from young mdx mice (a mouse model for muscular dystrophy disease) stimulate satellite cell differentiation, while FAPs from old mdx mice exerts the opposite effect. They showed a stronger pro-myogenic effect in response to histone de-

acetylase (HDAC) inhibitors treatment. Since HDAC inhibitors are associated with an increased expression of follistatin by FAPs, they proposed follistatin as a possible mediator in FAPs - satellite cell signaling (Mozzetta et al., 2013). In addition, FAPs have been shown to highly express IL-6 and IL-10, which are two important cytokines in the regulation of satellite cells and muscle regeneration (Joe et al., 2010) (Zhang et al., 2013) (Deng et al., 2012). Furthermore, in vitro co-culture experiments (cultures in transwell), showed also a positive effect of trophic factors produced by FAPs on the expansion and proliferation of satellite cells associated with myofibers (Fiore et al., 2016)

The supportive role of FAPs in muscle regeneration has been recently investigated by Fiore et al, by using an experimental model of FAPs ablation, which consists in FAPs inhibition by Nilotinib, a tyrosine kinase inhibitor with potent anti-fibrotic activity (Fiore et al., 2016). From this study two main functions can be ascribed to FAPs: the first one regards the regulation of ECM deposition; and the second one concerns their potential to sense signals and communicate with cells that are distant (paracrine effects). Indeed, using an in vivo model of acute injury, they demonstrate the relevant role of the “transient” release of ECM by FAPs and when this function is blocked it suppresses FAPs activity and expansion and the muscle do not regenerate properly. This failing in regeneration can be attributed to the influence of ECM in satellite cells activation, since the composition/amount of ECM influences the elasticity/stiffness of the satellite cells niche (Gilbert et al., 2010), as well as on the adhesion molecules availability to interact (Liu et al., 2011) (Wilschut et al., 2011) (Brohl et al., 2012).

Altogether, the muscle regeneration studies corroborate the importance of the cross-talk between satellite cells, FAPs and muscle myofibers to ensure a successful regeneration. The understanding of this multifaceted communication will help to identify key pathways important for the development of new tools to improve degenerative pathologies.

AIMS OF THIS THESIS

In the framework of this doctoral Thesis a collaborative work was established between Bongiovanni and Marazzi-Sassoon laboratories.

Muscle development and regeneration require the coordinated communication of multiple cell types. In the muscle stem cell niche different cell types work in concert to ensure the proper muscle function. A failure of reciprocal communication between these cells results in defective muscle regeneration.

The aim of this thesis was to gain insight into the intercellular communication taking place in the muscle stem cell niche. In the first part of my project, we studied the regulation of the release of skeletal muscle cell-derived extracellular vesicles using a murine myoblast cell line. In the second part, we examined the signals derived from tissue-resident mesenchymal stromal cells, the fibro-adipogenic progenitors and their functional role in the cross talk with muscle stem cells.

The specific aims of the doctoral Thesis presented herein are:

- To investigate the role of S-palmitoylation in modulating proteins involved in the biogenesis of extracellular vesicles and in the membrane organization of these skeletal muscle-derived vesicles. An integrated biochemical-biophysical approach was used for this purpose.
- To characterize the communication between fibro-adipogenic progenitors and primary satellite cells, focusing on the regulation of satellite cell fate. We used our in vitro experimental model to unravel the signals triggering satellite cell self-renewal. [Thesis Chapter II]

CHAPTER I

PALMITOYLATION IS A POST-TRANSLATIONAL MODIFICATION OF ALIX REGULATING THE MEMBRANE ORGANIZATION OF EXOSOMES

Palmitoylation is a post-translational modification of Alix regulating the membrane organization of exosomes

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ABSTRACT

Virtually all cell types have the capacity to secrete nanometer-sized extracellular vesicles, which have emerged in recent years as potent signal transducers and cell-cell communicators. The multifunctional protein Alix is a bona fide exosomal regulator and skeletal muscle cells can release Alix-positive nano-sized extracellular vesicles, offering a new paradigm for understanding how myofibers communicate within skeletal muscle and with other organs. S-palmitoylation is a reversible lipid post-translational modification characterized by the addition of an acyl chain to specific cysteine residues. It is involved in different biological processes, such as the trafficking of membrane proteins, achievement of stable protein conformations, and stabilization of protein interaction. Here, we have used an integrated biochemical-biophysical approach to determine whether S-palmitoylation contributes to the regulation of extracellular vesicle production in skeletal muscle cells. We ascertained that the structural organization of the lipid bilayer of the exosomal membrane with altered palmitoylation is qualitatively different compared to mock control exosomes. Furthermore, we showed that Alix is S-palmitoylated and that this post- translational modification influences its protein-protein interaction with CD9, a member of the tetraspanin protein family. We propose that S-palmitoylation regulates the function of Alix in facilitating the interactions among exosome-specific regulators, and maintains the proper structural organization of extracellular vesicle membranes.

INTRODUCTION

Extracellular vesicles (EVs) are a heterogeneous population of membranous particles, which have emerged in recent years as potent signal transducers and cell-cell communicators thanks to their content of proteins, lipids, and genetic material (Simons and Raposo, 2009) (Yanez-Mo et al., 2015) (Kowal et al., 2016). Based on the current knowledge of their biogenesis, EVs can be broadly divided into two main categories, exosomes and microvesicles, both involving membrane-trafficking processes. Until now, research on EVs has focused primarily on their role in the immune system and cancer. Recently, we have shown that skeletal muscle (SkM) cells can release Alix-positive exosome-like vesicles, which suggested a function for these EVs in SkM biology (Romancino et al., 2013). We now want to decipher how muscle cells generate these nano-vesicles and how this process is regulated.

Alix (also known as PDCD6IP) is an evolutionarily conserved, ubiquitously expressed, multifunctional adaptor protein (Odorizzi, 2006) (Matsuo et al., 2004) (Bissig and Gruenberg, 2014). By binding selectively to a variety of protein partners (i.e., components of the endosomal sorting complexes required for transport, ESCRT, actin and cortactin) and lipid (e.g., lysobisphosphatidic acid, LBPA), Alix has been implicated in many cellular processes, from endocytosis to cytoskeleton and membrane remodeling, and EV's biogenesis (Matsuo et al., 2004) (Bongiovanni et al., 2012) (Baietti et al., 2012) (Fisher et al., 2007) (Campos et al., 2016) (Mercier et al., 2016). The protein structure of Alix revealed a boomerang shape with a convex face (BRO1) encompassing a positively charged surface that might function as membrane bending domain (Kim et al., 2005). Alix functions in cellular events, such as cytokinesis, filopodia formation, membrane repair, viral and exosomal budding, that require the formation of negative membrane curvature and 100-nm scale membranous protrusions (Bongiovanni et al., 2012) (Morita et al., 2010) (Zhai et al., 2011b) (Jimenez et al., 2014). The subcellular architecture of SkM differs greatly from that of mononucleated cells. It is therefore predictable that preserving muscle structure and function requires a muscle-specific adaptation of known membrane trafficking and remodeling pathways

(Scheffer et al., 2014) (Charrin et al., 2013). For this reason, we and others have proposed that exosome biogenesis may differ according to the producing cell type and can also be sustained by direct exosome-like vesicles budding from the plasma membrane, as is the case of skeletal muscle, T cells and erythrocytes (Simons and Raposo, 2009) (Romancino et al., 2013) (Booth et al., 2006) (Hagerstrand H et al., 2006). Accordingly, highly ordered plasma membrane regions, such as lipid raft, tetraspanin-enriched microdomains (TEMs) and glycolipid-enriched membrane microdomains (GEMs), have been implicated in exosome biogenesis (Perez-Hernandez et al., 2013) (Thuma et al., 2016) (Yang et al., 2010). However, the membrane domain(s) responsible for exosome formation and structure are not clear, nor are the coupled regulatory mechanisms that control membrane remodeling.

Tetraspanins, a transmembrane protein family highly enriched in exosomal membranes, can form homodimers and heterodimers, and can interact with cholesterol, gangliosides, as well as transmembrane and cytosolic signaling proteins (Charrin et al., 2014) (Rana S., 2013). They are organized in clusters on the membrane, generating TEMs that have the capacity to facilitate vesicular fusion or fission (Hemler, 2008). It has been reported by Booth and coworkers that in lymphoblast and T cell lines plasma membrane domains enriched in tetraspanin (e.g., CD81, CD63) and endosomal lipids (e.g., LBPA), serve as platforms for exosome's budding (Booth et al., 2006). The same group also demonstrated that S-palmitoylation and myristoylation tags target a highly oligomeric, recombinant cytoplasmic protein to secreted vesicles (Shen et al., 2011). S-palmitoylation is a lipid post-translational modification (PTM), characterized by the attachment of a saturated fatty acid, palmitic acid (16:0), to specific cysteine residues of proteins (Blaskovic et al., 2013). Palmitoylation is distinguished from other lipid PTMs, such as prenylation and myristoylation, by its reversible nature. This PTM increases the hydrophobicity of proteins and regulates many key biological processes, such as localization, conformation and stability of proteins at membranes, and protein-protein interaction. In the case of tetraspanins (e.g., CD9), S- palmitoylation is required for their interactions and functions (Blaskovic et al., 2013) (Salaun et al., 2010) (Levental et al., 2010). However, the extent of the biological roles of this PTM remains to be fully elucidated.

Here, we have investigated whether S-palmitoylation regulates the membrane remodeling proteins involved in EV biogenesis in the context of TEMs, and the organization of exosomal membrane. We applied an integrated biochemical-biophysical approach to determine that S-palmitoylation modulates the integrity of SkM-derived exosome membrane and Alix' function.

MATERIAL AND METHODS

Comparative analysis of publicly available datasets

Data comparison and visualization were performed using R software (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org) with Bioconductor packages. UniProt accession numbers were assigned to the exosomal human protein dataset from ExoCarta in order to compare it with the S-palmitoylated protein dataset from SwissPalm. The intersection of the two datasets identified the exosomal proteins that are predicted to be S-palmitoylated. Fisher's exact test was used to confirm the different amounts of S-palmitoylation in the human and exosome proteomes. Gene ontology (GO) was used to annotate the S-palmitoylated proteins for cellular component.

Reagents and antibodies

Hydroxylamine solution (HAM), N-ethylmaleimide (NEM), BrijO20, and 2-bromohexadecanoic acid (2BP), were purchased from Sigma-Aldrich (St. Louis, MO, USA). EZ-LinkTM BMCC-Biotin, Dynabeads protein G, Pierce streptavidin HRP conjugated and goat anti-rabbit Alexa Fluor 680 were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Commercial antibodies included mouse anti-Alix for immunoblotting (clone 49/AIP1, BD Biosciences, San Jose, CA, USA) and anti-Alix for immunoprecipitations (clone 3A9, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-CD63, clone H-193 and antiCD9, clone KMC8.8 (Santa Cruz Biotechnology); anti-Hsp70/Hsc70 (Novus Biologicals, Littleton, CO, USA); anti-Bax (Sigma-Aldrich); anti-PARP (Cell Signaling Technology, Leiden, The Netherlands); anti-pan Actin, clone AC-40 (Sigma-Aldrich).

Extraction of Proteins from C2C12 cells and Alix Co-Immunoprecipitation

The mouse myoblast cell line C2C12 (ATCC-LGC, Wesel, Germany. ATCC® Number: CRL-1772TM) (Blau et al., 1985) was induced to differentiation with 2% horse serum in place of 15% FCS. After 2 days of differentiation, cells were cultured for one day with

or without 20μM 2BP. Cytoplasmic, membrane/organelle, nuclear, and cytoskeletal fractions were prepared using a ProteoExtract subcellular proteome extraction kit (Calbiochem, San Diego, CA, USA) according to the manufacturer's instructions.

For IP-ABE analysis, cells and EVs were lysed using lysis buffer (0.5% Triton X100, 50 mM Tris-HCl, pH 7.5, 5 mM NaCl, 2 mM $CaCl_2$, 3 mM KCl, 1mM $MgCl_2$, 100 mM sucrose), supplemented with complete EDTA-free Protease Inhibitor cocktail (Roche, Mannheim, Germany), phosphatase Inhibitor cocktails 2 and 3 (Sigma-Aldrich), and freshly prepared 50 mM N-Ethylmaleimide (NEM). The cell lysate was passed 8-9 times through a 26½ gauge syringe, gently shaken for 30 min at 4°C, and then was centrifuged at 16000g for 30 min at 4°C in order to remove insoluble material. Clear supernatant was collected, and protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific). IPs were performed using either 1 mg of cell lysates (Fig. 4A, B) or 0.1 mg of cell, microvesicles, or exosome lysates (Fig. 4C, D). For immunoprecipitation of the CD9-Alix complex C2C12 cells were lysed with hypotonic buffer, cellular debris and nuclei were removed, and supernatants were centrifuged at 100,000 g for 1 hour at 4°C. Membrane pellets were resuspended in Brij (Sigma-Aldrich, Old tradename BrijTM 98 V) buffer (1% BrijO20, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$), incubated by rocking for 30 minutes at 4°C, and centrifuged at 10,000 g for 10 minutes at 4°C. Supernatants were quantified by BCA protein assay. In all cases the volumes of lysate samples were corrected to have same protein concentration (2 mg/ml). Protein extracts were precleared adding 30μl of Dynabeads protein G to 1mg of proteins, and incubating under agitation for 1 hour at room temperature. Precleared supernatant was added to 3μg of monoclonal antibodies (anti-Alix, clone 3A9) and incubated by rocking overnight at 4°C. Subsequently, 30 μl of Dynabeads protein G were added to each sample, left under agitation for 2 hours at room temperature, and washed five times with ice-cold Brij buffer. After, 2x SDS-PAGE non reducing (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.004% bromophenol blue) or reducing (+ 5 mM DTT) sample buffer were added to samples, boiled and loaded on 8-16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE-SDS).

Acyl-biotin exchange (ABE) onto PVDF membrane

Immunoprecipitations were performed as described above with modifications. Briefly, precleared samples were incubated with anti-Alix (clones 3A9), and the immunoprecipitated proteins were washed five times with wash buffer containing 0.1%

IGEPAL® CA-630, 10 mM Tris-HCl, pH 7.5, 2 mM $CaCl_2$, 3 mM KCl, 1mM $MgCl_2$, previously kept on ice. After the last wash, 2x SDS-PAGE reducing sample buffer were added to the beads; the beads were then heated for 5 minute at 75-80°C. Samples were separated from magnetic beads, and loaded on an 8-16% PAGE-SDS, and electrophoresed at constant voltage (100 volts), using Tris-Glycine SDS running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS).

A modified ABE assay (Brigidi and Bamji, 2013) was performed on immunoprecipitated proteins immobilized on PVDF membranes. Briefly, each sample was split into two parts before loading on gels, one (1/3 of volume) was excluded from HAM treatment, and one (2/3 of volume) was treated with HAM. This measure was taken to normalize for protein degradation caused by HAM treatment. After electrophoresis, separated proteins were transferred onto 0.2 µm PVDF membranes (AmershamTM HybondTM, GE Healthcare Life Science, Waukesha, WI, USA). The membranes were stained with Ponceau S (Sigma-Aldrich), washed with buffer A (50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% IGEPAL® CA-630), and then incubated in buffer A supplemented with freshly prepared 20-50 mM NEM for 1 h. Subsequently, the membranes were cut and treated separately with HAM buffer (1 M HAM, 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.5% IGEPAL® CA-630) (+ HAM samples) or with buffer A (-HAM samples) for 1 h. After this step, all the membranes were washed 3 times with buffer B (50 mM HEPES, pH 6.2, 150 mM NaCl, 0.5% IGEPAL® CA-630) supplemented with 1 mM EDTA for 10 min, then membranes were incubated with Biotin buffer (0.3 mM BMCC-biotin in buffer B) for 1 h. Lastly, membranes were washed with PBS. A blocking buffer containing 3% BSA in TBS with 0.05% Tween-20 (TBST) was used to block the PVDF membranes, and after 2 washes with TBST, the re-assembled membranes (+ or –HAM) were incubated with streptavidin HRP conjugated diluted 1:20000 in blocking buffer. Subsequently, the membranes were washed once with blocking buffer for 30 min, and 3-4 times with TBST for 20 min. In order to detect the originally palmitoylated protein bands, the membranes were incubated for 5 min using a chemiluminescent substrate (Pierce ECL western blotting substrate, Thermo Scientific), and then were exposed for 1-5 min to Amersham HyperfilmTM ECL (GE Healthcare Life Science).

Western Blotting

PVDF membranes were probed with primary antibodies, and after washing steps were incubated with Alexa Fluor 680 (Invitrogen, Life Technologies, Paisley, United Kingdom) anti-rabbit or IRDye 800 (LI-COR Biosciences, Lincoln, USA) anti-mouse

IgGs. To determine signal intensity from infrared bands we used an Odyssey Infrared Imaging System (LI-COR) and the LI-COR imaging software. Intensity of the Ponceau bands was quantified using ImageJ software and used to normalize signals from specific antibodies.

EV isolation

EVs were isolated and quantified according to a previously published method (Romancino et al., 2013). This isolation method included a penultimate centrifugation step in Eppendorf polypropylene conical tubes (10,000 x g for 30 min at 4°C, in Eppendorf rotor F-34-6-38) that allowed the removal/isolation of larger microvesicles (μVes). Subsequently, nano-sized EVs, comprised mainly of exosomes, were pelleted in Beckman Coulter polypropylene open top tubes (118,000 x g for 70 min at 4°C, in Beckman rotor SW28). After washing, the pellet was resuspended either in RIPA buffer or in PBS, for further immunoblotting or biophysics analyses, respectively. To estimate the amount of secreted vesicles, we quantified and compared the total protein content of the vesicle lysates using the BCA assay.

Analysis of size distribution by Light Scattering

Scattered light intensity and its time autocorrelation function g2(t) were measured simultaneously on different vesicle samples at the temperature T=20 °C by using a Brookhaven BI-9000 correlator (Brookhaven Instruments, Holtsville, NY, USA), equipped with a solid-state laser tuned at λ0 = 532 nm. The samples were diluted to a final total protein content of 33 and 66 μg/ml for exosomes and microvesicles, respectively, in order to avoid vesicle interaction and multiple scattering artefacts. Apart from an instrumental parameter ß, g2(t) directly yields the field correlation function g1(t) (g2(t)=1+ß|g1(t)|2), which is analyzed to determine the weight averaged distribution aq(σ) of hydrodynamic diameters σ : g1(t) = $\int$ aq(σ) exp{-D(σ)q2t} d σ . In the latter expression, q=4πñλ0-1sin(θ/2) is the scattering vector, with θ=90° being the scattering angle and ñ=1.3367 the medium refractive index, and D(σ) is the diffusion coefficient, related to σ by the Stokes-Einstein relation: D(σ)=kBT(3πησ)-1, where kB is the Boltzmann constant, T is the temperature and η is the solvent viscosity (Ross-Murphy, 1977) aq(σ) is here shaped as a simple two-parameter gamma function with average σ0: aq(σ)dσ=Γ(α)-1ααD(σ0)-αD(σ)α- 1exp{-αD(σ)D(σ0)-1}dD(σ). Absolute values for scattered intensity (Rayleigh ratio) were obtained by normalization with respect to

toluene, whose Rayleigh ratio at 532 nm was taken as $28 \cdot 10^{-6}$ cm^{-1}. Intensity absolute values were used to estimate the total content of exosomes and microvesicles.

Since light scattered intensity is proportional to the square of the mass of scatterers, the measured size distribution is dominated by larger mass objects. Therefore, while the maximum of the distribution indicates the most abundant species in terms of mass and mass concentration, the most numerous species are certainly included in the range between the increase of the size distribution and its maximum.

Atomic Force Microscopy Images

Vesicles samples were diluted to a total protein content of 1μg/ml in PBS and a 30 μl drop was deposited on a freshly cleaved mica substrate for 10 minutes at room temperature. Vesicles images were immediately acquired by using an Asylum Research Cypher Microscope (Oxford Instruments, Abingdon, United Kingdom) operating in liquid tapping mode (resolution 512 × 512, scan rate 0.5 Hz). We used Olympus BL-AC40TS cantilevers with a typical radius of curvature of 10 nm (nominal spring constant 0.1 N/m, resonance frequency 25 kHz in liquid). The free oscillation amplitude was set at 10 nm and images were acquired at 50% larger than free amplitude. AFM experiments were performed at the PSCM facility of the European Synchrotron Radiation Facility (Grenoble, France).

SAXS and SANS analyses

The conceptual experiment and theory is the same for X-rays and neutrons, differences are the physics of the X-ray (electro-magnetic radiation) versus neutron (neutral particle) interactions with matter. Measurement is of the coherent scattering from the sample. Because objects (e.g., exosome and microvesicles) in solution are randomly oriented, the scattering pattern represents an average of the scattering from all possible orientations. Hence, in measurements the scattering intensity recorded on the 2D detector will not depend on the direction of the scattering vector, but only on its magnitude, the momentum quantum $q = (4\pi \sin\theta)/\lambda$, where 2θ is defined as the scattering angle, and λ is the wavelength of the incoming X-ray beam. The 2D images are the integrated curves of scattering intensity I vs. q, called scattering profiles, where q is given in units of inverse nanometer. The scattering contribution of the vesicle on its own is produced by subtracting the buffer scattering profile from the vesicle-solution scattering profile. This background-subtracted profile is the starting point for the analysis of solution SAXS and SANS data. A wealth of structural information can be

gained from such profiles, including the organization of the lipid bilayer (Bouwstra et al., 1993) (Hirai et al., 2003) (Castorph et al., 2010) (Chen et al., 2009) (Nickels et al., 2017). In the case of objects such as vesicles, the X-ray scattering of the whole vesicle appears at low momentum transfer, enabling the characterization of the vesicle size and shape. In contrast, the scattering intensity at high q (around q=1 nm^{-1}) provides the information about the bilayer organization.

Small Angle X-ray Scattering (SAXS) data were collected at different synchrotron facilities: beamline SWING of Synchrotron Soleil, Gif-sur-Yvette, France; beamline SAXS of synchrotron Elettra, Trieste, Italy; beamline BM29 of European Synchrotron Radiation Facility (ESRF), Grenoble, France. In all cases, exosome and microvesicle samples in PBS from different preparations were frozen at -20°C. The day before the SAXS/ small angle neutron scattering (SANS) analyses samples were thawed, pooled, and suspended in PBS at total protein content between 0.5 and 1.0 mg/ml. Samples were injected in a 1 mm optical path capillary kept at 25 °C by using the respective injection methods (automatic autosampler at ESRF, automatic injection with air bubble separating different volume of the sample at Soleil; static manual loading at Elettra). Several (seven to ten) consecutive measurements of 60 seconds were performed for each sample by moving the sample under the beam. At the Elettra, where the sample could not be moved each single acquisition lasted 10 s. Since successive exposures showed the same scattering pattern, indicating no radiation damage, the data were averaged over the different exposures. The buffer was measured before and after each measurement by using the same capillary. Detector images were radially integrated to obtain the scattering pattern I(q). Small Angle Neutron Scattering data were collected at beamline D16 of Institute Laue Langevin (ILL), Grenoble, France. The sample was resuspended in fully deuterated PBS, incubated for 48 hours at 4°C, and loaded into 1 mm optical path quartz cuvette. Measurements were performed with an average duration of 20 minutes by using different sample detector distance, covering different ranges of scattering vector q.

Statistical Analysis

Data were expressed as mean + S.D. and were evaluated using Student's t test. Mean differences were considered statistically significant when p values were less than 0.05 (*).

RESULTS

S-palmitoylated proteins are enriched in the exosome subcellular compartment

To evaluate the extent to which S-palmitoylation is functionally linked to exosome biogenesis, we first conducted a comparative analysis of publicly available data. We used the SwissPalm manually curated protein S-palmitoylation database (http://swisspalm.epfl.ch), which encompasses 1160 S-palmitoylated, high-confident (HC) hits from palmitoyl-proteome screens, and the ExoCarta exosome database (http://www.exocarta.org), which includes information on exosomal human protein from diverse cell types and body fluids (Blanc et al., 2015) (Keerthikumar et al., 2016). The overlap of these two datasets showed that a surprisingly high number of HC S-palmitoylated proteins are annotated in ExoCarta as exosomal human proteins (Fig. 14A).

Furthermore, although 10% of the human proteome undergoes S-palmitoylation (Sanders et al., 2015), and only 5.5% of the HC S-palmitoylated proteins are distributed in the human proteome, there is about a three-fold enrichment in the number of these proteins in the exosomal protein content (p value<0.001, Fisher's exact test) (Fig. 14B).

We then investigated the distribution of S-palmitoylated proteins across different cellular compartments using gene ontology (GO) annotation (Ashburner et al., 2000) (Cheung et al., 2016) (Gene Ontology, 2006) for cellular components. Consistent with the previous findings, exosomes contained the highest percentage of S-palmitoylated proteins (80.7%), compared to all the other cellular compartments (Fig. 14C).

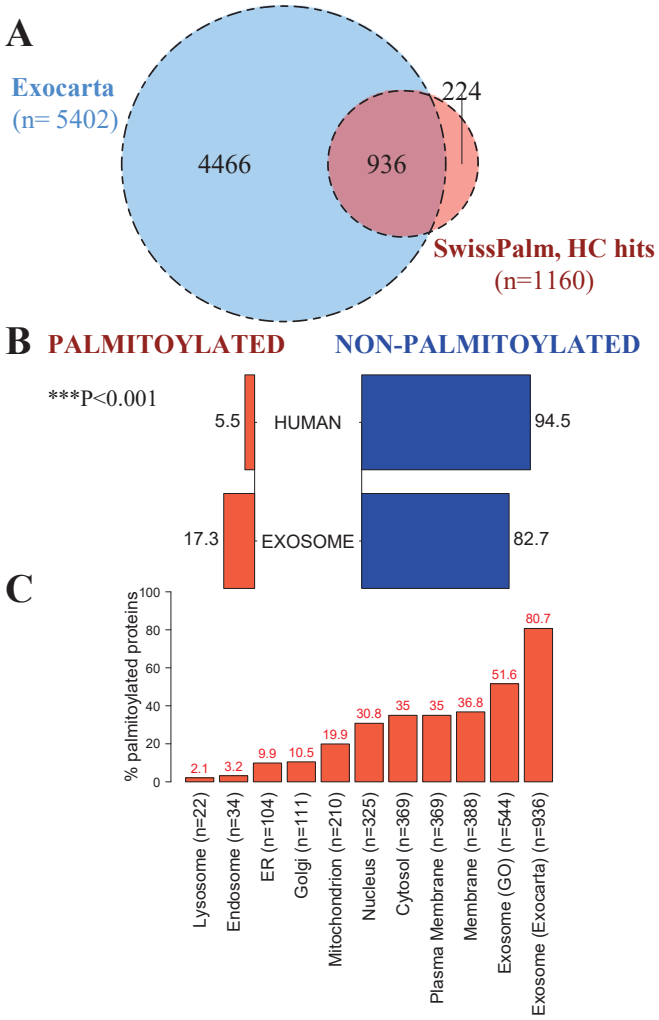


Figure 14. Bioinformatic analyses of S-palmitoylation in exosomes. (A) Venn diagram representing the overlap between exosomal human proteins from ExoCarta and S-palmitoylated proteins with high confidence (HC) from SwissPalm. (B) Pyramid plot showing the different distribution of S-palmitoylation between the human proteome and exosomal protein content (Fisher's exact test) (p value<0.001). (C) Distribution of S-palmitoylation hits in the different cellular compartments.

Analysis of the organization of skeletal muscle-derived EV membranes

To evaluate the organization of the exosomal membrane, we performed synchrotron radiation SAXS experiments on exosomes and microvesicles derived from C_2C_{12} myotube cultures, either untreated or treated with 2-bromopalmitate (2BP), an inhibitor of the acylation/deacylation protein machinery. For this purpose, we used C_2C_{12} myotubes at day 3 of differentiation. Treatment with 20 µM 2BP for 24 hours did not induce any overt morphological changes in the myotubes compared to untreated cells

(Fig. 15A). Moreover, the myotubes remained viable, as demonstrated by the levels of the apoptotic markers BAX and PARP that remained unchanged in the cytoplasmic and nuclear fractions of treated and untreated myotubes, respectively (Fig. 15B). EVs, including larger microvesicles and exosomes, were isolated from myotubes by sequential ultracentrifugation; ultracentrifugation is the most common EV purification technique, and it includes a penultimate centrifugation step (10,000g) that allows the removal/isolation of larger microvesicles for the subsequent pelleting of nano-sized EVs (118,000g), commonly called exosomes (Gardiner et al., 2016) (Thery et al., 2006). Consistently, we will use this definition of exosome and microvesicles in this paper.

Following treatment with 2BP, microvesicles and exosomes were isolated from myotubes and subjected to SAXS analyses (see Materials and Methods section for detail). In all measurements, we observed a peculiar scattering profile at high q (around $q=1\ nm^{-1}$); the scattering profile was different comparing mock control exosomes with either microvesicles or 2BP-treated exosomes (Fig. 16A). Intriguingly, the same structural feature was observed on different sample preparations and by using different beam lines (SAXS Elettra and SWING Soleil) (Fig. 16A). This finding was remarkable given that we were analyzing a complex natural system with no external control regarding the actual composition of vesicles.

To gain further information on the nature of this structural feature, we performed combined SANS and SAXS experiments by using the same sample preparations, namely the myotube-derived exosomes (Fig. 16B). Our SANS results show that, in fully deuterated buffer, the hump typical of SAXS patterns disappeared indicating that the main difference among exosome and either microvesicles or 2BP-treated exosomes can be ascribed to the structure of the lipid bilayer (Fig. 16B).

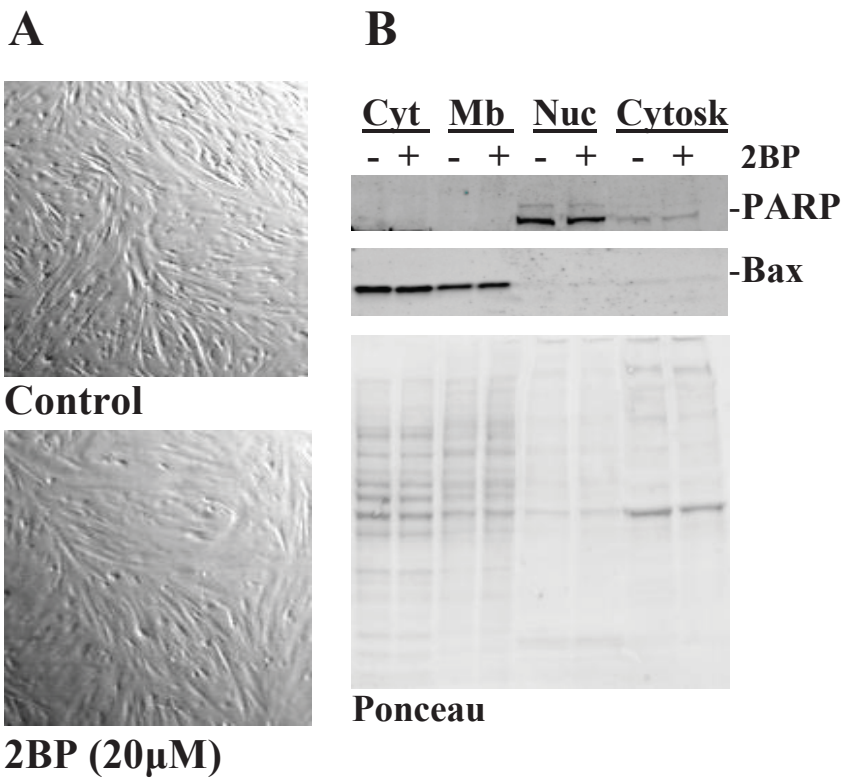


Figure 15. 2BP treatments did not induce apoptotic signs in C2C12 myotubes. (A) Cell morphology is unaltered by 2BP treatment (20 μM, 24 hours). (B) The levels of the apoptotic markers BAX and PARP in cytoplasmic and nuclear fractions, respectively, of untreated and 2BP-treated C_2C_{12} myotubes (day 3 of differentiation) are comparable.

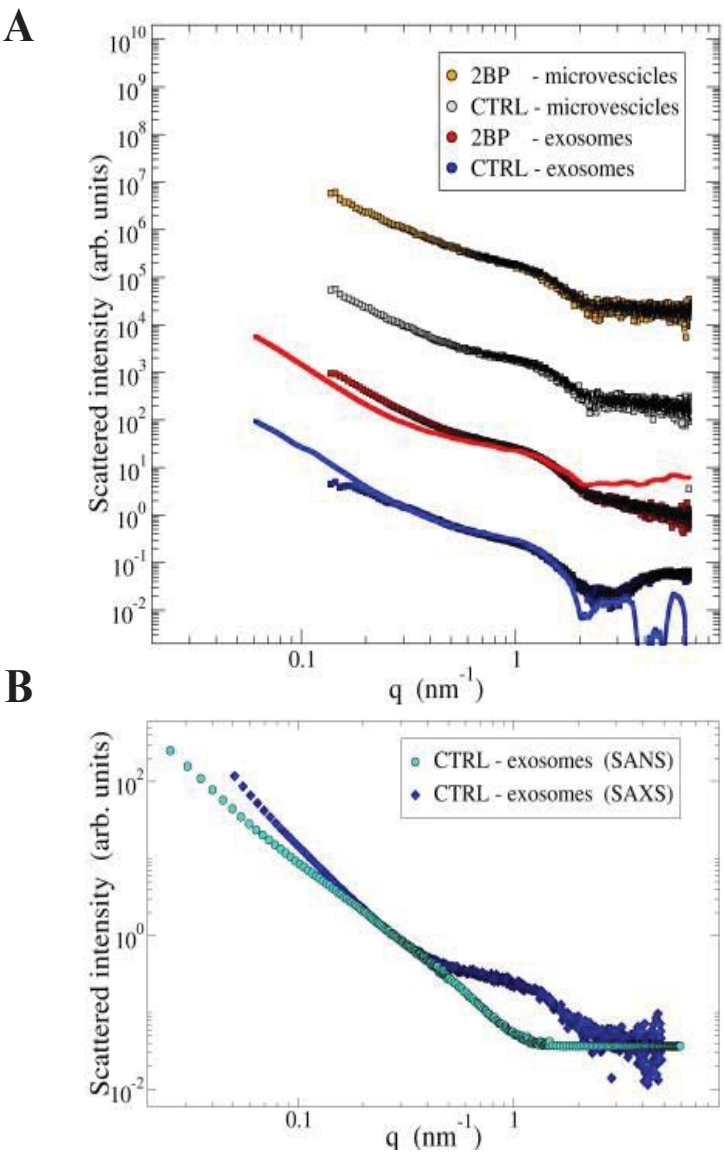


Figure 16. Analysis of the membrane organization of exosomes and microvesicles, and the influence of S- palmitoylation inhibition. Exosomes and microvesicles treated or not with 2BP were analyzed by SAXS and SANS; the patterns of different samples are color-coded as in legends. Patterns are vertically shifted for clarity. (A) SAXS analyses performed at Elettra (squares), and Soleil (solid lines) showing a qualitatively different scattering pattern of control exosomes compared to microvesicles and 2BP-treated exosomes. (B) SAXS (diamonds) and SANS (circles) analyses of control myotube-derived exosomes.

The inhibition of palmitoylation affects exosome number and size distribution

To examine the effects of blocking S-palmitoylation on the number and size distribution of exosomes we applied atomic force microscopy (AFM) and dynamic light scattering (DLS) analyses on myotube-derived vesicles. Using AFM we measured the size of myotube-derived exosomes in solution; figure 17A shows the typical image of an exosome with an approximately spherical shape, an apparent width of w'~200 nm and an apparent height of h~70 nm, in line with previous electron microscopy measurements of fixed exosomes (Romancino et al., 2013). The actual width w was estimated as ~125 nm by applying a standard correction for a parabolic tip on a step w=w'-(8Rtiph)0.5, where Rtip~10nm is the curvature radius of the AFM tip (Podesta et al., 2006).

The analyses of 2BP-treated and untreated myotube-derived nano-sized vesicles by DLS showed for both vesicle preparations an average hydrodynamic diameter of about 150 nm that is consistent with the size of exosomes (Fig. 17B). In addition, 2BP treatment generated a different size distribution of vesicles: we noticed an increase in the number of vesicles of larger size (inset in Fig. 17B). DLS analyses revealed also a significant increase in the number of exosomes following 2BP treatment (Fig. 17C), data that were corroborated by an increase in the total protein concentration of 2BP-treated exosomes, compared to control (Fig. 17D). These observations indicate that, the alteration of palmitoylation by 2BP induced a general large secretion of myotube-derived nano-sized vesicles and a slight increased proportion of larger vesicles than mock control microvesicles.

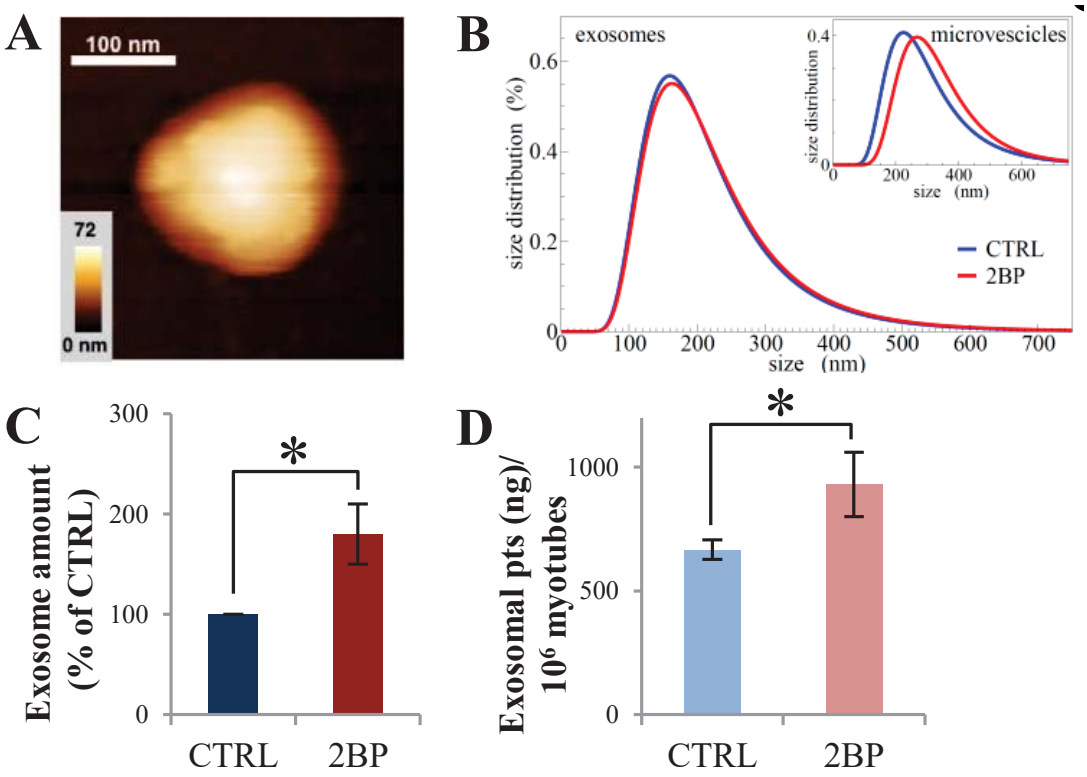


Figure 17. Inhibition of S-palmitoylation influences the number and size distribution of exosomes. (A) Atomic Force Microscopy image of a typical exosome. The solid white bar and the colored scale in the inset indicate the width and height of the pictured vesicle, respectively. (B) Size distribution of control (blue) and 2BP treated (red) nano-sized vesicles was measured by dynamic light scattering (DLS). Control (blue) and 2BP treated (red) microvesicles are shown in the figure inset. 2BP treatments induce a significant increase of exosomes number (C) and exosomal protein concentration (D). (C) The number of exosomes was determined by DLS. (D) The relative exosome protein content was determined by BCA assay. Results are presented as average + SD of three independent experiments,*p<0.05.

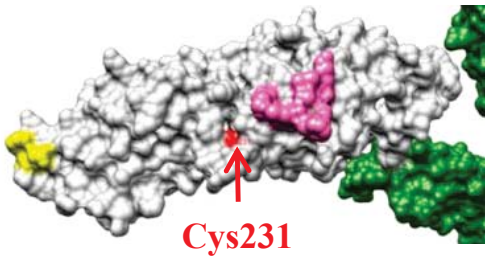
Endogenous Alix is palmitoylated in skeletal muscle cells

Next, we focused on one of the most abundant protein of exosomes, Alix, and we determined whether it would undergo S-palmitoylation. For this purpose, we first queried the palmitoyl-proteomic data reported in Swiss Palm for Alix S-palmitoylation and found four palmitoyl-proteome studies, in which both human and mouse Alix were reported as S-palmitoylated proteins (Ren et al., 2013) (Hernandez et al., 2016) (Serwa et al., 2015) (Chesarino et al., 2014). We therefore used the bioinformatics tool, SeqPalm (Li et al., 2015), to identify cysteine in position 231 of HuAlix as the predicted

residue to undergo S-palmitoylation (Fig.18). To investigate whether Alix is S-palmitoylated in differentiated SkM cells and SkM-derived EVs, we developed a modified immunoprecipitation-Acyl-Biotin Exchange (IP-ABE) method (Brigidi and Bamji, 2013) on immunoprecipitated Alix, immobilized on polyvinildifluoride (PVDF) membranes. This assay uses hydroxylamine (HAM) to cleave palmitate from immunoprecipitated proteins, exchanging it with biotin and allowing for the detection of palmitoylated proteins by streptavidin. Because HAM treatment usually degrades part of the immunoprecipitated proteins, we performed it on immunoprecipitated Alix immobilized on PVDF membranes, rather than in solution, in order to minimize protein loss. We then compared the levels of palmitoylated-Alix among different samples by quantifying the palmitoylated-Alix band (detected by streptavidin-HRP) and the total Alix band (detected by anti-Alix antibodies), and then by calculating the ratio of Palm-Alix/Alix. This modified, semi-quantitative assay allowed for the analysis of the endogenous palmitoylated-Alix in cell and EV lysates. Following treatment with 2BP, myotubes were lysed and the endogenous Alix was immunoprecipitated using an anti-Alix monoclonal antibody. The modified IP-ABE assay showed that endogenous Alix was S-palmitoylated in myotubes, as evidenced by the streptavidin-positive bands in IP-ABE samples, which was absent in the control lanes where the essential ABE reagent hydroxylamine was omitted (Fig. 19A). Furthermore, altering palmitoylation with 2BP significantly reduced the palmitoylation level of Alix confirming that the endogenous protein carries this PTM (Fig. 19A, B).

We then investigated whether exosomal Alix was also palmitoylated within myotube-derived exosomes. Equal amounts of exosome-, microvesicle- and total cell-lysates were immunoprecipitated using anti-Alix antibody, and ABE assays were performed on the immunoprecipitated material. Comparing the ratio of palmitoylated Alix versus the total Alix immunoprecipitated from the three lysates, we found that the levels of palmitoylated Alix were significantly higher in exosomes than in microvesicle- or total cell-lysates (Fig. 19C, D). Thus, we can conclude that exosomal Alix is palmitoylated to a larger extent than cellular Alix.

A



B

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PDC6I_HUMAN   AKLANQAADYFGDAFKQCQYKDTLPK-----EVFPVLAAKHCI 263
PDC6I_MOUSE   AKLANQAADYFGDAFKQCQYKDTLPK-----EVFPTLAAKQCI 263
PDC6I_XENLA   AKLANQAADYYGDAFKQCQYKDTLSK-----EVFPILAAKHCI 262
PDC6I_RAT     AKLANQAADYFGDAFKQCQYKDALPKYFYFQEVFPTLAAKQCI 264
Q5ZJ70_CHICK AKLANQAADYYGDAYKQCQYKDTLPKYFYFQEVFPVLAAKHCI 262
Q7SY03_DANRE AKLANQAADYYGDAFKQCQYKENLPK-----DVLPVLAAKHCM 259
              **********:***:*****: * *     :*:* ****:*:
```

Figure 18. In silico analysis to evaluate S-palmitoylation of Alix protein. (A) Structure of Alix (PDB identity code 2OEV). Human Cys231 (red) is exposed and localized on the convex surface of the Bro1 domain, in close proximity to the membrane interacting site (pink). The Bro1 region, responsible for the autoinhibition of Alix, is also shown (yellow). (B) Multiple sequence alignment of the Bro1 region, encompassing HuCys231, of Alix orthologues from the indicated species. Human Cys231 (red) is conserved in vertebrates.

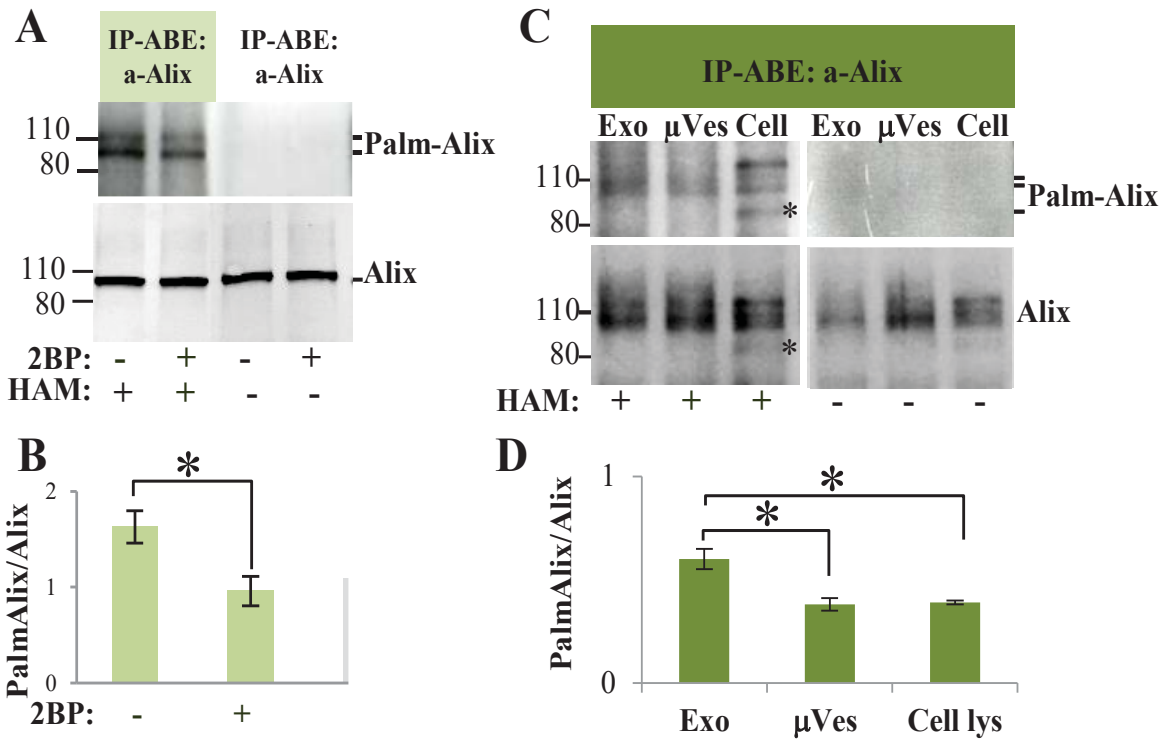

Figure 19. Alix is S-palmitoylated in skeletal muscle cells, and the S-palmitoylation level of exosomal Alix is higher compared to cellular and microvesicle Alix. (A) A representative IP-ABE assay showing S-palmitoylation of the endogenous Alix in cellular extracts of C2C12 myotubes, omission of hydroxylamine (-HAM) is shown as a control. IPs were performed using 1 mg of cell lysates. A decreased level of Alix palmitoylation is observed after 2BP treatment. (B) Quantification of S- palmitoylation by densitometric scanning of Palm-Alix (detected by streptavidin-HRP) and Alix (detected by anti-Alix immunoblot) bands; the ratio of Palm-Alix/Alix was calculated in three independent experiments. (C) A representative blot of IP-ABE assay showing higher levels of palmitoylated Alix in myotube-derived exosomes (Exo) compared to microvesicles (µVes) and cellular (Cell) extracts, isolated from C2C12 myotubes. IPs were performed using 0.1 mg of lysates. Omission of HAM is shown as a control. The asterisk indicates the cleavage product of Alix. (D) Quantification by densitometric scanning of Palm-Alix (detected by streptavidin-HRP) and Alix and Alix cleavage product (detected by immunoblot); the ratio of Palm-Alix/Alix was calculated in three independent experiments. Results are presented as average + SD, *p<0.05.

Alix interacts with CD9 and 2BP affects their binding

It has been shown previously that several members of the tetraspanin protein family are S-palmitoylated and regulate protein sorting into exosomes (Perez-Hernandez et al., 2013). We therefore wanted to assess the distribution of Alix and the tetraspanin family members, CD9 and CD63, in myotube-derived exosomes after 2BP treatment.

Treatment with 2BP induced a significant reduction of both CD9 and Alix monomers and at a larger extent homodimers in exosomes (Fig. 20A, B). We also found a slight reduction of Alix and CD9 monomers after palmitoylation inhibition with 2BP, compared to untreated cells (Fig. 20A, B). In contrast, the CD63 levels increased, while HSP70 and actin remained unchanged (Fig. 20A, B). Because of the similar behavior of Alix and CD9 and the role of TEMs in exosome biogenesis, we asked whether Alix would take part in the tetraspanin web via interaction with CD9. We found that the CD9 and Alix endogenous proteins co-precipitate from myotube membranes extracted with the Brij98 detergent (Fig. 20C, D), which preserves protein interactions within the TEMs (Charrin et al., 2003). This result identifies Alix as a novel interactor of CD9 in the TEMs. Furthermore, we found that the interaction between the two proteins depends on their palmitoylation status, because 2BP-altered palmitoylation resulted in a significant reduction of Alix-CD9 binding (Fig. 20C, D). Indeed, although similar amounts of Alix were immunoprecipitated from control and 2BP-treated myotube extracts, we detected a reduced amount of CD9 upon treatment with 2BP (Fig. 20C, D), indicating that S-palmitoylation supports the interaction among Alix and CD9, as it occurs for tetraspanin complexes in TEM.

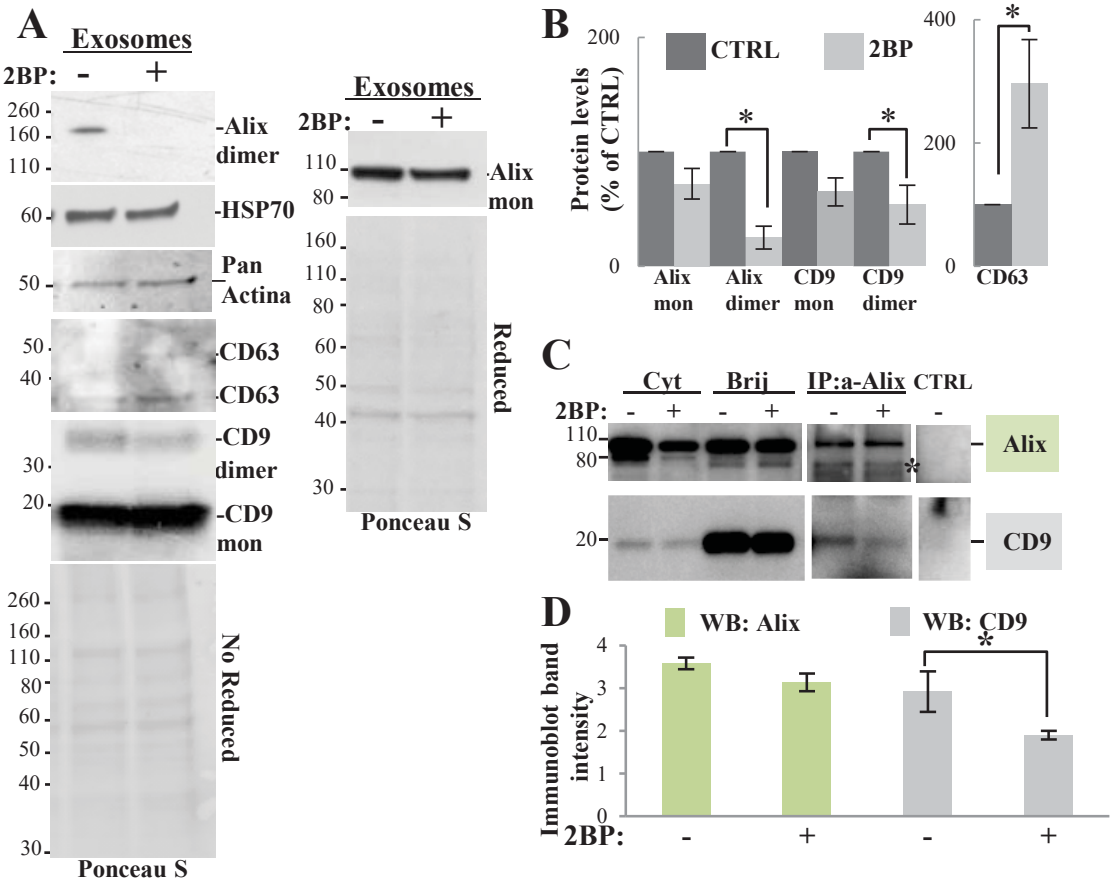

Figure 20. S-palmitoylation influences cargo distribution of exosomes and Alix-CD9 interaction. (A) Representative immunoblots of exosome lysates showing that 2BP treatment induced a significant reduction of Alix and CD9 and an increase of CD63 levels. (B) Quantification of immunoblots by densitometric scanning; band signals were normalized to the protein levels determined by Ponceau S staining. (C) Representative immunoblots of cytosol (Cyt) and Brij98-soluble membranes (Brij), and the IPs of Brij extracts using anti-Alix antibody. The asterisk indicates cleavage products of Alix. Alix and CD9 interact, and the palmitoylation inhibition (+2BP) affects their binding. (D) Quantification of Alix and CD9 bands in immunoblots of IPs. Results are presented as average + SD of three independent experiments, *p<0.05.

DISCUSSION

Protein S-palmitoylation is a reversible post-translational lipid modification that is involved in different biological processes. Here, we applied an integrated biochemical-biophysical approach to examine whether S- palmitoylation modulates Alix function and maintains the integrity of exosomal membranes. First, by a comparative analysis of publicly available data we determined a higher percentage of palmitoylated proteins in exosomes, compared to cellular proteome. This is in line with in vitro data showing that a prenylation/palmitoylation tag can target a recombinant, highly oligomeric cytoplasmic protein to exosomes (Shen et al., 2011). These findings suggest that this PTM could be a distinctive signature for exosomal proteins.

The enrichment of S-palmitoylated proteins in exosomes is similar to what occurs within the core complexes of caveolae and TEM microdomains (Yang et al., 2010). TEM membrane platform have been implicated in exosome biogenesis and virus assembly and are characterized by a liquid-ordered membrane structure, also induced by virtue of palmitoylation-mediated tetraspanin clustering (Perez-Hernandez et al., 2013) (Thuma et al., 2016) (Yang et al., 2010). Recently, it has been shown that SAXS analyses can be used to describe the structure of the lipid bilayer of synaptic vesicles or vesicles of synthetic origin (Bouwstra et al., 1993) (Hirai et al., 2003) (Castorph et al., 2010). Thus, to investigate the functional significance of the enrichment of S-palmitoylated proteins in exosomes with respect to the organization of the native exosomal membrane we performed synchrotron radiation SAXS experiments. Under our experimental condition, we found a qualitatively structural difference among myotube-derived exosomes and microvesicles, as indicated by the comparison of their SAXS scattering intensity at high q (around q=1 nm^{-1}); the same difference was also

observed among exosomes derived from mock control and 2BP-treated myotubes. To gain further information on the nature of this structural feature, we performed combined SANS and SAXS experiments by using myotube- derived exosomes. To our knowledge, SANS studies have been previously performed using model membrane systems; the only two SANS analyses of biological membranes were performed on detergent-resistant membrane (DRM) vesicles and Bacillus subtilis membranes (Chen et al., 2009) (Nickels et al., 2017). Intriguingly, our SANS results clearly show that in fully deuterated buffer, the SAXS scattering pattern typical of the exosome preparation disappeared. In our experimental condition, SANS pattern does not highlight the contrast among the different vesicle components (protein, lipid, nucleotides) and the fully deuterated solvent. On the other hand, the electron density contrasts between vesicle components and the solvent are different among them, and hence are detectable in SAXS measures. Thus, we can confidently ascribe the SAXS scattering pattern to the structure of a lipid bilayer, confirming what has been found in other systems (Castorph et al., 2010). A more detailed analysis, in terms of typical onion shell models and SANS analyses with contrast variation, is ongoing but is beyond the scope of the present work. Here, we prefer to exploit the main qualitative features fostered by these data in a model-free approach. SAXS data indicate a characteristic membrane organization of exosomes that is significantly different from that of microvesicles and 2BP-treated exosomes; we related these qualitative differences to changes in the organization of the lipid bilayer. This result corroborates and expands recent data, based on EV staining with a fluorescent, polarity-sensitive lipid probe, which showed that exosomal membranes are characterized by a high membrane lipid order compared to microvesicles (Osteikoetxea et al., 2015). Interestingly, high membrane lipid order is typically found in membrane nano- and micro-domains (e.g., TEMs, lipid rafts) at the immunological synapses and at sites of cell adhesion, viral entry, or viral budding. The distinct membrane organization of exosomes compared to that of microvesicles may represent a unique feature underlying exosomes' role in intercellular signaling and/or biogenesis. Hence, S-palmitoylation, which influences both the membrane organization and number of exosomes may be a key PTM of their biogenesis.

Alix is a bona fide membrane remodeling protein prone to dimerizing and oligomerizing (Fisher et al., 2007) (Pires et al., 2009) (Munshi et al., 2007), and it is one of the most abundant components of exosomes. Thus, we focused on this exosome key player to examine whether S-palmitoylation modulates also its function. Although palmitoylation,

unlike other types of lipidation, does not require a distinct sequence motif in the substrate, it has been proposed that a 51 amino acid (AA) protein fragment centered on a cysteine encompasses a latent motif that serves as a palmitoylation site (Li et al., 2015). By using a bioinformatics tool, SeqPalm (Li et al., 2015), we identified the predicted palmitoylated cysteine residue (HuCys231); notably, Cys231 in human Alix is exposed on the protein surface in close proximity to the hydrophobic loop, which is important for membrane binding (Matsuo et al., 2004), and is surrounded by a 51 AA fragment highly conserved across vertebrates (Fig. 18). Furthermore, we determined that endogenous Alix is S-palmitoylated in myotubes; the combined observations that the largest fraction of palmitoylated Alix segregated with exosomes and that palmitoylation inhibition with 2BP drastically reduced the amount of Alix dimer in exosomes, point to a key role of palmitoylation in driving Alix transfer to these nanovesicles, possibly by regulating Alix dimerization, as it does for tetraspanins (Charrin et al., 2014). Similarly to other tetraspanin family members, dimeric Alix represents the active form of the protein that interacts with protein partners (e.g., ESCRT-III CHMP4 polymers) and localizes to membranes; the Alix dimer functions as a scaffolding protein during membrane remodeling processes, and disruption of Alix dimerization inhibits the release of HIV (Charrin et al., 2003). Because of the similar behavior of Alix and tetraspanin and the role of TEMs in exosome biogenesis, we asked whether Alix would take part in the tetraspanin web via interaction with CD9. From our results, we were able to infer that endogenous Alix interacts with CD9 and S-palmitoylation supports this interaction, as it also does for tetraspanin complexes in TEMs. To date, the only PTMs known to regulate the structure and function of Alix are ubiquitination and phosphorylation (Bongiovanni et al., 2012) (Sun et al., 2016) (Schmidt et al., 2005) (Dores et al., 2015). Furthermore, our group demonstrated that the binding of OZZ, the substrate binding component of the RING type ubiquitin ligase complex OZZ-E3, changes Alix's conformation and makes the protein accessible for further ubiquitination (Bongiovanni et al., 2012). Our current results suggest that also S-palmitoylation regulates Alix interaction to CD9 and the localization of Alix in exosomes.

Taken together, our results provide insights into the role of S-palmitoylation in maintaining a proper exosome membrane structure organization, in addition to regulating the proper localization of Alix in SkM exosomes and supporting the interactions among exosome-specific regulators/biomarkers (i.e., Alix and CD9). Moreover, beyond its biological relevance, our study also provides the means for a comprehensive structural characterization of EVs.

REFERENCES

Please see references at *REFERENCE Section*.

CHAPTER II

REGULATION OF SATELLITE CELLS SELF-RENEWAL MEDIATED BY FIBRO-ADIPOGENIC PROGENITORS

Regulation of satellite cells self renewal mediated by fibro-adipogenic progenitors

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Manuscript ongoing

ABSTRACT

Successful skeletal muscle regeneration is mediated by coordinated cellular events, involving the main actors, the muscle stem cells (called satellite cells) and the other components of the muscle stem cell niche. Within the niche, tissue-resident mesenchymal stromal cells, named fibro-adipogenic progenitors (FAPs), are known to play a key role during regeneration, establishing a cross talk with satellite cells, as they have been proposed to promote the myogenesis of activated satellite cells. During muscle regeneration, satellite cells are activated and start to proliferate; some of them give rise to differentiated cells, other self-renew to replenish the pool of stem cells. The cell fate determination of satellite cells is the result of intrinsic and extrinsic signals coming from different components of the stem cell niche, but the specific signals and the mechanism driving satellite cells self-renewal are poorly understood. Here we investigated the communication between FAPs and satellite cells in the context of satellite cell fate decision. Here, we found that FAPs conditioned media promote satellite cells self-renewal. We noticed that satellite cells exposed to FAPs conditioned media grew in an anchorage independent manner, forming aciniform clusters of cells, linked on underlying myotubes. Our data reveal that the FAPs conditioned media enhanced the number of Pax7+ MyoD- satellite cells compared to control, a molecular signature found in quiescent satellite cells in vivo. We observed that satellite cells amplified in culture were mostly Pax7+/MyoD+ before the exposition to FAPs conditioned media. These results might indicate the influence of FAPs to let satellite cells return to a Pax7+ MyoD- state after a phase of proliferation. We have used conditioned media depleted of extracellular vesicles, thus these data suggest that FAPs release soluble factors able to regulate satellite cells self-renewal properties. It has been already shown that several pathways are involved in satellite cells quiescence such as Notch, Wnt and BMPs. Here, we observed that satellite cells self-renewal mediated by FAPs conditioned media was suppressed by the addition of

Noggin, a specific inhibitor of the BMPs/activin pathway. These data suggest that a member of the BMP family might be released by FAPs and triggers satellite cells self-renewal. This work brings new insights in the regulation of satellite cells self-renewal mechanism and the role of FAPs communication in the niche.

INTRODUCTION

Skeletal muscle is able to regenerate following injury. Satellite cells are the progenitor cellular source of new muscle however proper muscle regeneration is dependent upon other resident cell types including the immune and vasculature system and fibro-adipogenic progenitors (FAPs). FAPs are mesenchymal stromal cells that play a supportive role during regeneration through sustaining the formation of new muscle fibers with the release of extracellular matrix (Fiore D. et al 2016) and promoting terminal differentiation of activated satellite cells through paracrine signaling (Joe et al., 2010).

Satellite cells are able to differentiate as well as self-renew to replenish the satellite cell pool. Satellite cell fate is governed by intrinsic and extrinsic factors present within the stem cell microenvironment or niche. Different interactions (i.e. from the basal lamina and the myofiber) lead to a polarization of satellite cell resulting in different cell fate of the daughter cells (Bentzinger et al., 2012). It has been shown that specific pathways signal through Notch (Mourikis et al., 2012) and the Sprouty homologue 1 (SPRY1 (Shea et al., 2010) to maintain the quiescence of satellite cells, however it is not completely unraveled the regulation of this mechanism as well as the role of other cell types of the niche in this control. Here, we investigated the potential role of FAPs in the regulation of satellite cell self-renewal. We used media conditioned by FAPs to study the impact of FAPs-derived signals on isolated satellite cells. In our in vitro model, we showed that FAP-conditioned media (depleted of extracellular vesicles) can regulate satellite cell competence and promote stem cell self-renewal. Altogether, our results suggest that FAPs secrete soluble factors directing satellite cell fate and point to the potential molecular pathways involved.

MATERIAL AND METHODS

Mice

Satellite and FAPs cells were FACS-sorted from 3 month old C57BL/6JRj mice (n=42) for in vitro analysis. For RNA sequencing, 3 months old C57Bl6J *PW1*^{IRESnLacZ} transgenic mice were used (n=21) (Besson et al., 2011). Mice were sacrificed by cervical dislocation. All work with mice was carried out in adherence to French government and European guidelines.

Fluorescence-activated cell sorting analysis

For fluorescence-activated cell sorting (FACS), limb muscles from 3 month old C57Bl6J mice were minced and digested in Hank's Balanced Salt Solution (HBSS, Gibco) containing 2 µg ml−1 collagenase A (Roche), 2.4 U ml−1 dispase I (Roche), 10 ng ml−1 DNase I (Roche), 0.4 mM CaCl and 5 mM MgCl as described previously (Besson et al., 2011; Mitchell et al., 2010). Primary antibodies at a concentration of 10 ng ml−1 were: rat anti-mouse Cd45-PeCy7 (eBiosciences), rat anti-mouse Ter119-PeCy7 (BD Biosciences), rat anti-mouse Cd34-BV421 (BD Biosciences), rat anti-mouse Sca1-FITC (eBiosciences), rat anti-mouse PDGFRα-PE (eBiosciences), rat anti-mouse Cd31-BUV737 (BD Biosciences) rat anti-mouse α7integrin-A700 (RD biosystem) and rat anti-mouse Cd11b-BUV395 (BD Biosciences). Cells were incubated for 30 minutes on ice and washed once with ice-cold HBSS, filtered and re-suspended in HBSS containing 0.2% (w/v) bovine serum albumin (BSA), 1% (v/v) penicillin-streptomycin and 10 ng ml−1 DNase I. Cells were incubated with the viability marker 7-AAD (BD bioscience) for 10 minutes at 4°C prior to cells sorting. Flow cytometry analysis and cell sorting were performed on a FACS Aria (Becton Dickinson) with appropriate Fluorescence Minus One controls. PDGFRα+ cells were sorted by the expression of Cd34, Sca1, PDGFRα and negatively selected for Ter119, Cd45, Cd11b,

α7integrin and Cd31. Satellite cells were sorted by the expression of α7integrin, Cd34 and negatively selected for Ter119, Cd45, Cd31, PDGFRα and Sca1. For RNA sequencing of satellite cells, PDGFRα+ and PDGFRα- cells limb muscles from 3 month old C57Bl6J *PW1*^{IRESnLacZ} transgenic reporter were minced and digested as described above. Primary antibodies at a concentration of 10 ng ml−1 were: rat anti-mouse CD45-PeCy7 (eBiosciences), rat anti-mouse TER119-PeCy7 (BD Biosciences), rat anti-mouse CD34-E450 (eBiosciences), rat anti-mouse SCA1-A700 (eBiosciences), rat anti-mouse PDGFRα-PE (eBiosciences). PDGFRα+ cells were sorted by the expression of C12FDG, CD34, SCA1, PDGFRα and negatively selected for TER119 and CD45. PDGFRα- cells were sorted by the expression of C12FDG, CD34, SCA1 and negatively selected for TER119, CD45 and PDGFRα. Satellite cells were sorted by the expression of CD12FDG, CD34 and negatively selected for TER119, CD45, and SCA1.

Primary cell culture

Cells from limb muscles of 3 months mice were obtained as described above. Immediately after sorting, satellite cells were plated at a density of 2000 cell per cm^2 in gelatin coated wells, and grown in amplification medium (AM) (high-glucose Dulbecco's modified eagle medium (DMEM, Gibco), 20% heat inactivated FBS (Invitrogen), 10% heat-inactivated horse serum (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-Glutamine (Gibco) and 1% (v/v) Na-pyruvate (Gibco) supplemented with 2.5 ng/ml bFGF (Invitrogen). FAPs, immediately after sorting, were plated at a density of 1000 cell per cm^2, and grown in amplification medium (AM): high-glucose Dulbecco's modified eagle medium (DMEM, Gibco), 20% heat inactivated FBS (Invitrogen), 10% heat-inactivated horse serum (Gibco), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-Glutamine (Gibco) and 1% (v/v) Na-pyruvate (Gibco). Media was changed every two days.

Cells were cultured under normoxic conditions (21 % O_2) or hypoxic conditions (1.5 % O_2) using a hypoxia incubator chamber (Stem cells technologies) and an oxygen sensor probe (GasBadge Pro, Industrial scientific) that display oxygen concentration in real-time. Noggin (R&D system) was used at the final concentration of 50 ng/ml.

To isolate mouse skin fibroblasts, dorsal skin from 3 months old mice was collected and fat was removed with a razor blade. The skin was incubated in trypsin-EDTA 0.25% at 37°C during 2h in order to remove the epidermis and skin explants of approximately 4 mm^2 were placed in 24 wells plate. The medium was changed every 2

days and skin explants were removed from the wells after a week, when fibroblasts have grown attached have attached to the plate.

Conditioned media preparation

For conditioned media preparation, FAPs were grown in AM described above for one week, until 70% confluence was reached. Then cells were gently wash with PBS and incubated for 48 hours in high-glucose Dulbecco's modified eagle medium (DMEM, Gibco), 20% exo-depleted FBS (System Bio), 1% (v/v) penicillin-streptomycin (Gibco), 1% (v/v) L-Glutamine (Gibco) and 1% (v/v) Na-pyruvate (Gibco).

Media were harvested, processed with differential centrifugation to separate extracellular vesicles (exosomes and microvesicles) as described in (Romancino et al., 2013). This EVs isolation method included a penultimate centrifugation step at 10,000 x g for 30 min, that allowed the removal of larger microvesicles, and a subsequent centrifugation at high speed (118,000 x g for 70 min at 4°C) to separate the exosomes. The resulting EV-depleted FAPs conditioned media were filtered (0,22um) to ensure sterility and used on satellite cells (freshly prepared or as frozen aliquots).

Immuno-staining

Cells were fixed in 4% (w/v) paraformaldehyde and processed for immunostaining as described previously (Mitchell et al., 2010). Primary antibodies were: anti-Pax7 (Developmental Studies Hybridoma Bank), anti MyoD (Santa Cruz), anti-MF20 (Developmental Studies Hybridoma Bank). Antibody binding was revealed using species-specific secondary antibodies coupled to Alexa Fluor 488 (Molecular Probes), Cy3 or Cy5 (Jackson ImmunoResearch). Nuclei were counterstained with DAPI (Sigma).

RNA-Sequencing

Satellite cells, PDGFRα+ and PDGFRα- cells were isolated by FACS from hind limb muscle from 3 months *PW1*^{nLacZ} mice. We purified RNA from muscle stem cell population using RNAqueous - Micro total RNA isolation Kit (life-technologies) with a gDNA degradation step. Directional libraries were prepared using Truseq Stranded mRNA sample preparation kit following the manufacturer's instructions (Illumina). Libraries were checked for concentration and quality on DNA chips with the Bioanalyser Agilent (Illumina).

The libraries were quantified 593 by fluorimetric measurements with the Qubit® dsDNA HS Assay Kit (ThermoFisher). 51-bp Single Read sequences were generated on the

Hiseq2500 sequencer according to manufacturer's instructions (Illumina). The multiplexing level was 2 samples per lane. Reads were cleaned of adapter sequences and low-quality sequences using an in-house program (https://github.com/baj12/clean_ngs). Only sequences at least 25 nucleotides in length were considered for further analysis. Tophat version 1.4.1.1 (Trapnell et al., 2009), with default parameters, was used for alignment on the reference genome (GRCm38 from Ensembl database version 74). Genes were counted using HTSeq-count version 0.6.1 (Anders et al., 2015) (parameters: -t exon -i gene_id -m intersection603nonempty -s yes).

RESULTS AND DISCUSSION

Satellite cells grow in an aciniform clustered morphology in response to FAP-conditioned media

FAPs have been proposed to support myogenesis through paracrine effects (Joe et al., 2010) as well as to be a source of small extracellular vesicles (EVs) (Sandonà M, 2017). In order to explore the cross-talk between FAPs and satellite cells, we evaluated the impact of FAPs conditioned media and FAPs derived EVs on satellite cells phenotype in vitro. We started by producing three different products from sorted FAPs, harvested after 2 days of culture: i) complete FAPs-conditioned media (completed with EVs, referred as FAPs CM); ii) EV-depleted, FAP-conditioned media (EV-depleted FAPs CM); iii) FAP-derived EVs (FAP-EVs) (Figure 21). Satellite cells were sorted following the strategy described in figure 22 and cultured for 3 days in amplification media, followed by incubation with the FAPs conditioned media (FAPs CM and EV-depleted FAPs CM), FAP-derived EVs, or the un-conditioned media as control (Figure 23)

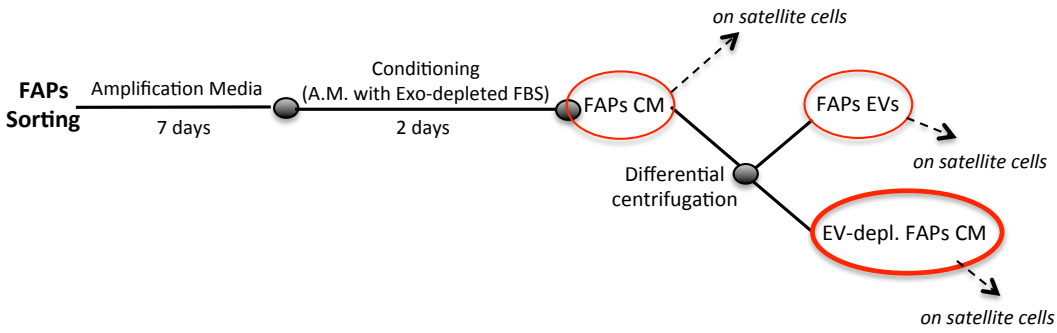

Figure 21. Schematic representation of FAPs culture and production of three FAP derived products (red circles). After 48h of conditioning, FAPs conditioned media (FAPs CM) is harvested; part of it is used on satellite cells, part is processed by differential centrifugations to separate the EV fraction and the EV-depleted FAPs CM, both used on satellite cells subsequently.

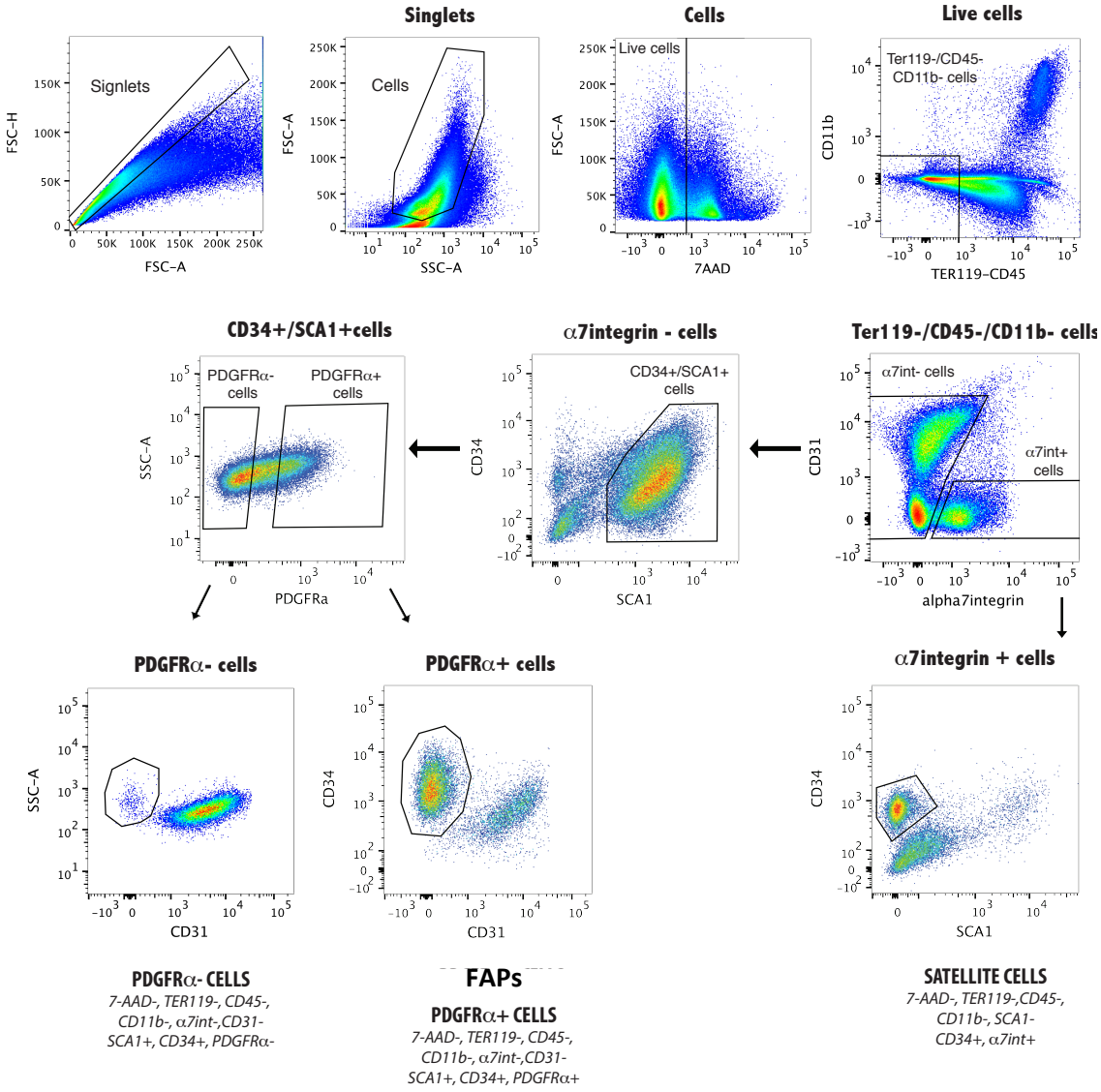

Figure 22. Cell sorting strategy using specific surface markers to isolate FAPs and satellite cells. Forelegs muscles from C57Bl/6 mouse were dissected and digested with a cocktail of collagenase and dispase for 90 minutes at 37°C. Live cells were selected with the viability marker 7-AAD. Satellite cells were selected by the negative expression of CD45, Ter119, CD11B, Sca1 and the positive expression of CD34 and α7-integrin. FAPs were selected by the negative expression of CD45, Ter119, CD11B, α7-integrin, CD31 and the positive expression of Sca1, CD34. PDGFRα+. Adapted from (Ollitrault D, 2017).

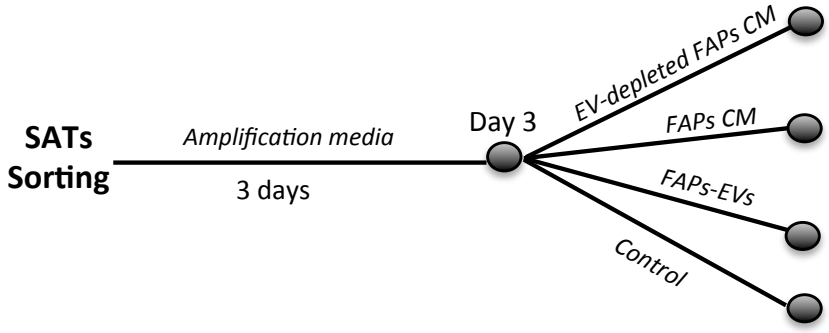

Figure 23. Schematic representation of satellite cells (SATs) culture and treatment with FAPs derived products. Freshly sorted satellites cells were culture for 3 days in amplification media (+bFGF), then incubated with FAPs conditioned media (FAPs CM and EV-depleted FAPs CM) and the same media un-conditioned supplied with FAP-EVs or not (control).

In the course of these experiments, we observed a specific and striking morphology of the growth and organization of satellite cells following exposure to FAPs conditioned media (Figure 24) referred to as aciniform. In contrast, our preliminary data showed no phenotypically relevant difference in satellite cells cultured with FAPs derived EVs (data not shown). The aciniform morphology was induced by FAP-conditioned media with or without the EV fraction suggesting that EVs were not the central players. Therefore, we performed all further analyses using FAPs conditioned media EV-depleted (EV-depleted FAPs CM). As shown in more detail in figure 25b and c, in presence of EV-depleted FAPs CM, satellite cells grew forming 3D clusters of round cells (either Pax7+ and/or MyoD+) that can vary in size between 5-50 cells per cluster (Figure 25d), and that showing an aciniform morphology we will designate therefore from now on as grapes. In the presence of control media, cells attached to the plate and started to differentiate into myotubes, acquiring an elongated shape (Figure 25a).
The satellite grapes were typically attached to a myotube cell (Figure 25c) and were enriched for Pax7+MyoD- cells that would indicate a quiescent state (Figure 25c). Quiescent satellite cells (Pax7+MyoD-) are mainly found in in vivo resting muscle (Zammit et al., 2006). In response to injury, satellite cells undergo activation and differentiate to ensure proper muscle regeneration. During the activation process, satellite cells express MyoD together with Pax7 and become Pax7-MyoD+, when committed to differentiation (Zammit et al., 2006). To our knowledge, we described for the first time a culture model system that keep Pax7+MyoD- satellite cells in 3D grapes in culture for at least three days.

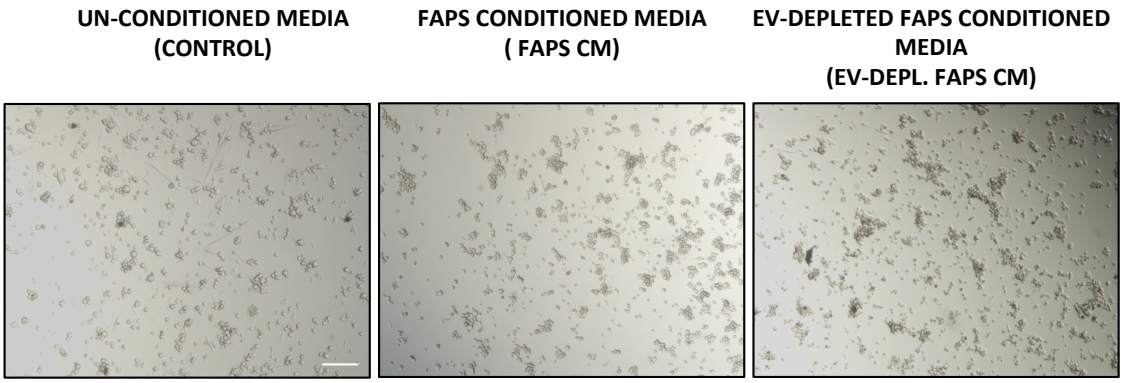

Figure 24 Effects of different FAPs conditioned media on satellite cells morphology. Freshly sorted satellite cells after 3 days of amplification were incubated with FAPs conditioned media (complete of EVs), EV-depleted FAPs conditioned media (deprived of FAPs derived EVs) and the same media un-conditioned as control; scale bar 100μm.

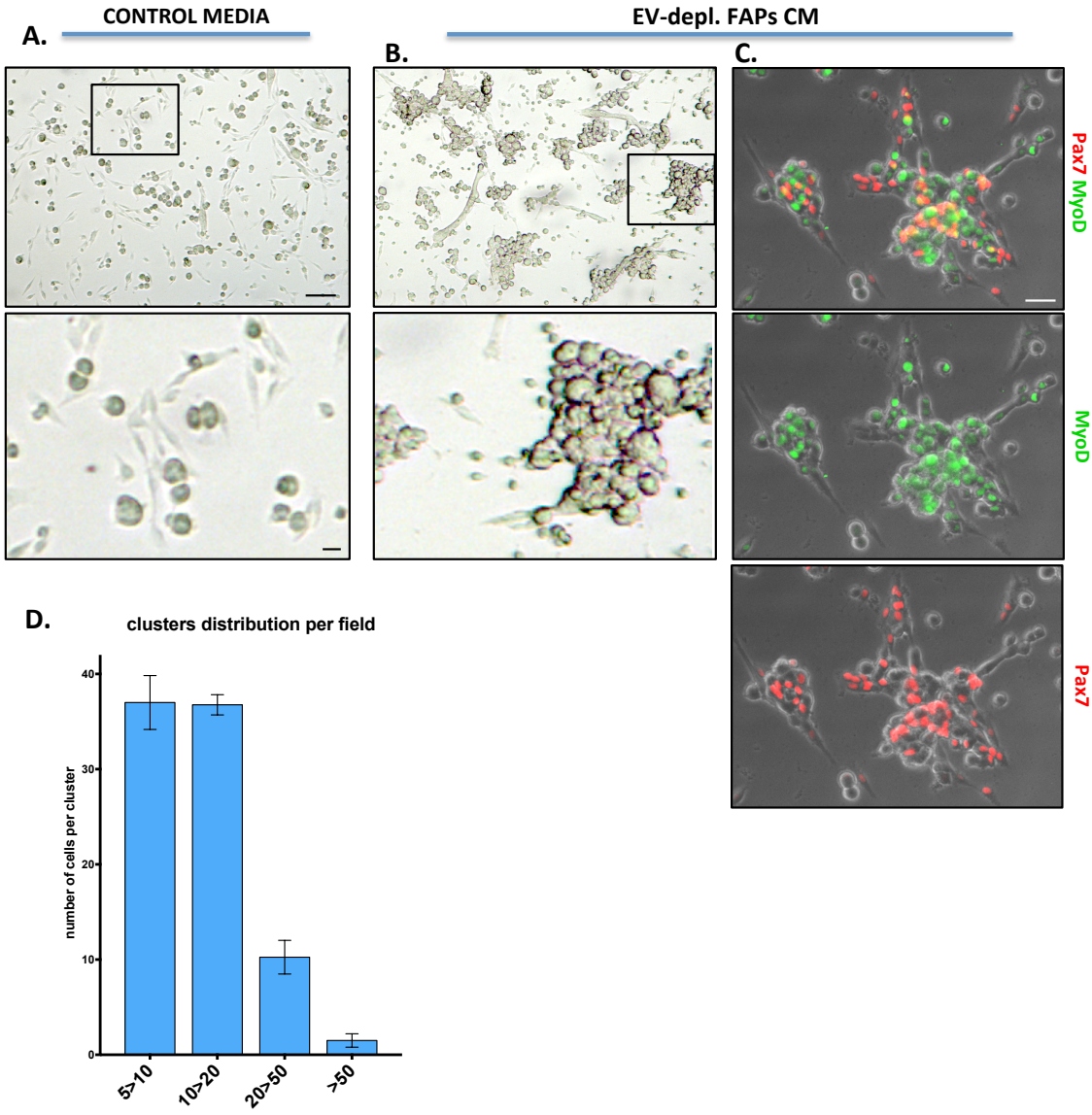

Figure 25. Satellite cells grow forming clusters in EV-depleted FAPs conditioned media. The effect of EV-depleted FAPs conditioned media (CM) (B) on satellite cells compared to control (A). Satellite cells have been freshly FACS sorted and amplified during 3 days with amplification medium and treated the next 3 days with EV-depleted FAPs CM; scale bar 50µm on the top, 10µm on the bottom. (C) Higher magnification of a cluster of satellite cells in EV-depleted FAPs CM, stained for Pax7 (red) and MyoD (green); scale bar 50µm. (D) Quantification of clusters containing from 5 to 50 cells in EV-depleted FAPs CM.

FAPs-conditioned EV-depleted media enriches grapes in Pax7+MyoD- satellite cells in vitro

Satellite cells are sensitive to changes in their microenvironment and the isolation and separation from the niche is sufficient to promote cell cycle entry (Machado et al., 2017). Indeed, at day 3 of in vitro cultivation, proliferating cells were mainly Pax7+MyoD+ (activated satellite cells, 91%), whereas Pax7+MyoD- cells were very rare, representing only 1% of the total cell number (Figure 26). After treatment with EV-depleted FAPs CM or control media for three more days in culture (day 6), we observed a 40-fold increase in the percentage of Pax7+MyoD- cells with EV-depleted FAPs CM media and only 9-fold increase with control media, as compared with day 3 in culture (amplification medium) (Figure 26). We also noticed that the percentage of Pax7-MyoD+ cells was quite similar between the two conditions, while Pax7+MyoD+ (double positive) population was represented at a lower level in EV-depleted FAPs CM condition (Figure 26).

These data showed a clear effect of EV-depleted FAPs CM in the induction of the satellite cells Pax7+MyoD-. Looking at the total number of cells, we observed that EV-depleted FAPs CM stimulated a massive increase (3 times more) in satellite cells number from day 3 to 6, as compared to the control (Figure 27). These results indicate that EV-depleted FAPs CM, and thus FAPs soluble factors, act not only in the satellite cell self-renewal (higher number of Pax7+MyoD-) but also on satellite cells proliferation. The enrichment in Pax7+MyoD- cells observed in our culture condition (three days in amplification media followed by three more days in EV-depleted FAPs CM) can be explained as result of two possible mechanisms. First, it can be the result of a great expansion of the un-committed Pax7+MyoD- satellite cells already present in culture. Second, Pax7+MyoD- cells can derive from the transitory subset of Pax7+MyoD+ cells, highly represented at day 3; among this transitory subset and reversible activated state, some cells would down-regulate MyoD and return to a Pax7+MyoD- state, leading to a return to a more “primitive” stage (Figure 28).

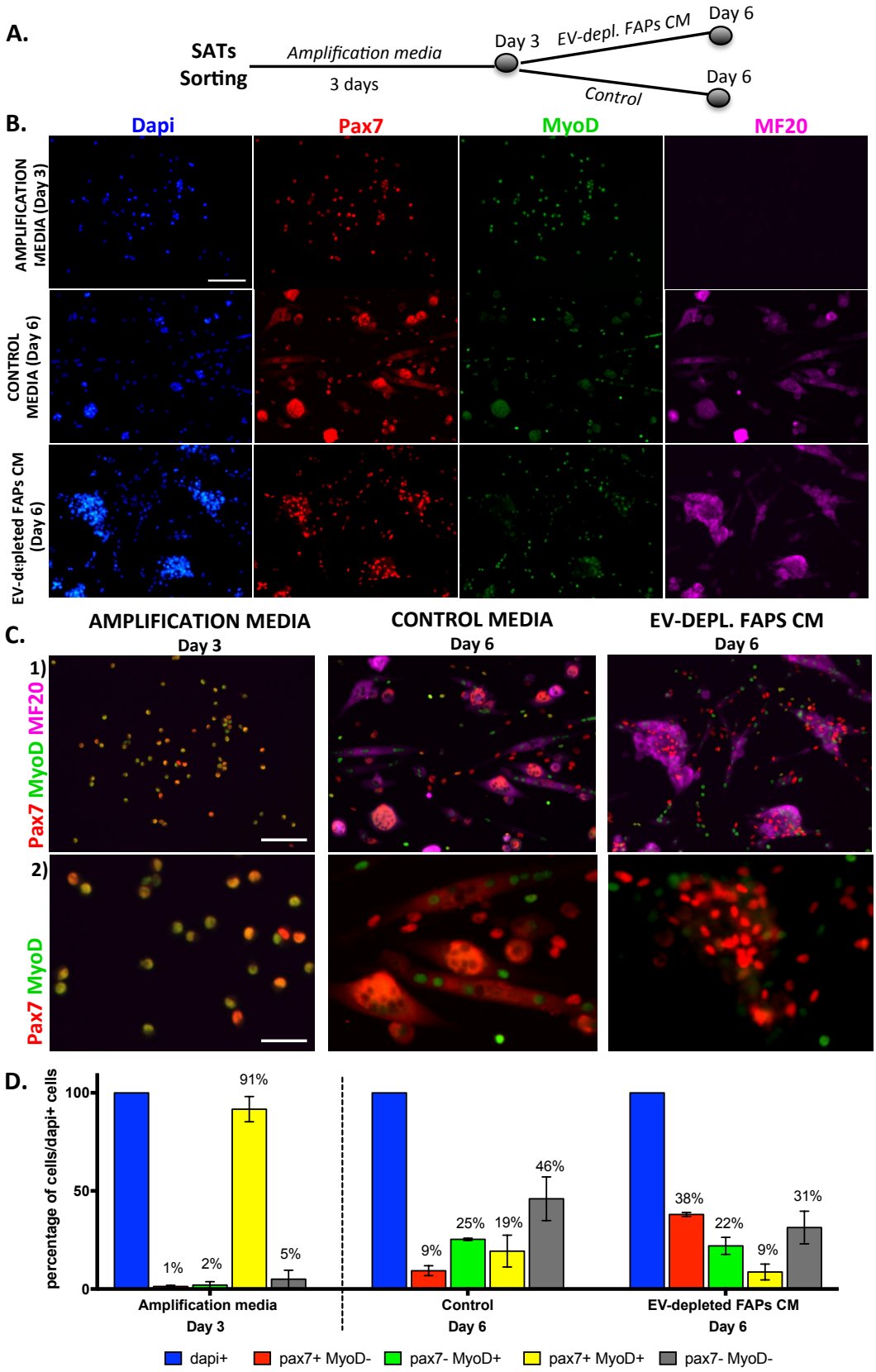

Figure 26. EV-depleted FAPs CM enhances the number of Pax7+/MyoD- cells. (A) Schematic representation of the experimental plan. (B) Staining of satellites cells at day 3 (in amplification media) and at day 6 (in EV-depleted FAPs CM or un-conditioned media as control) stained for Pax7 (red), MyoD (green) and MF20 (magenta); scale bar, 100µm. (C) Higher magnifications of satellite cells, stained; scale bar, 1) 100µm 2) 50 µm. (D) Quantification of the percentage of cells on total cell number of dapi+ nuclei.

With the data presented here, these two scenarios cannot be discerned or their co-existence excluded. Consistent with our data, the ability of MyoD+Pax7+ cells to return to a divergent fate becoming Pax7+MyoD- has been already studied both in immortalized C212 cells (Yoshida et al., 1998) and in satellite cells cultured with freshly isolated myofibers (Zammit et al., 2004). However, the mechanism and the signals involved in the self-renewal process are still poorly understood. Here, we showed the enrichment and peculiar distribution of Pax7+MyoD- cells in grapes rising from newly formed myotubes (MF20+) (Figure 26). Zammit and colleagues, analyzed satellite cells cultured with myofibers and suggested that the myofiber and the cluster architecture, including the presence of some MyoD+ cells in the cluster, have a crucial role in maintaining the ability of activated satellite cells (Pax7+/MyoD+) to get back to a Pax7+MyoD- “stem” state (Zammit et al., 2004). In our model, we observed a similar phenomenon with an increase of the Pax7+MyoD- signature after exposition to EV-depleted FAPs CM, as well as the consistent association of satellite cells grapes (3D cluster) with small myotubes. Thus, both extrinsic and intrinsic factors might be involved in the regulation of satellite cell fate decision. It has been reported for instance that Angiopoietin 1, through ERK pathway activation, enhances Pax7 expression and supports the undifferentiated state, in an autocrine/paracrine fashion (Abou-Khalil et al., 2009). On the other hand, it has been shown that the activation of Sprouty homologue 1, SPRY1), a negative regulator of ERK signaling, is necessary for the return to quiescence of a subpopulation of myoblasts (Shea et al., 2010).

Taken together, our results might be important to understand the mechanisms that guide satellite cells self-renewal, as well as the regulation of cell fate adoption after regenerative process.

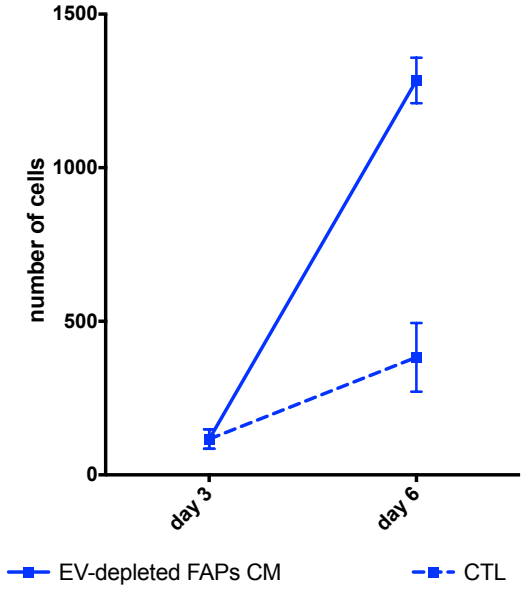

Figure 27. EV-depleted FAPs CM enhances the number of satellite cells. Quantification of dapi+ cells at day 3 (after three days of culture in amplification media with bFGF) and at day 6 (after three more days in culture with EV-depleted FAPs CM (continuous line) or with the un-conditioned media as control (dashed line)) .

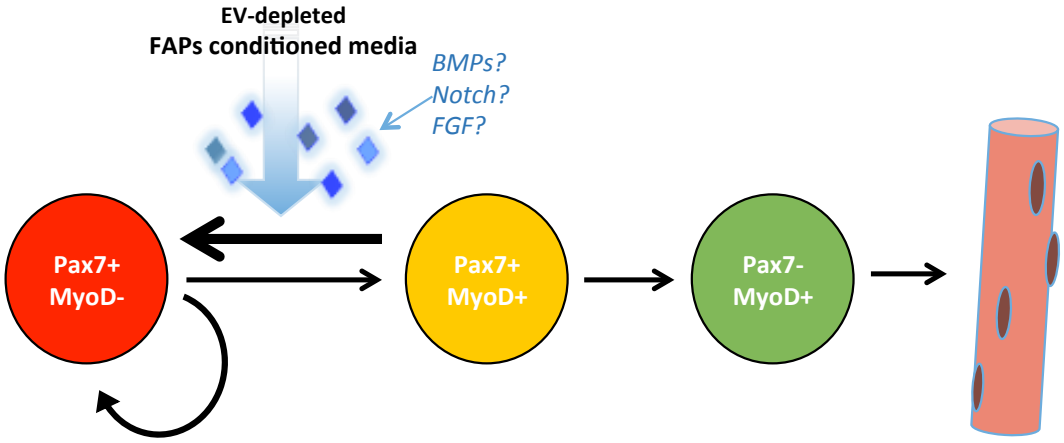

Figure 28. Schematic representation of the model hypothesized for the promotion of the state Pax7+ MyoD- in satellite cells. EV-depleted conditioned media could act enhancing the expansion of the present pool (Pax7+ MyoD-) or could act influencing the transitory population of activated satellite cells (Pax7 + MyoD+) to return to the more primitive state.

Hypoxia does not affect the number of Pax7+/MyoD- cells

It is known that oxygen tension levels represent an important signal to ensure stem properties in the stem cell niche (Liu et al., 2012). Moreover after an injury, when the regenerative process takes place, some area of the muscle become temporally hypoxic due the disruption of the tissue, including the capillary system (Ollitrault D, 2017). Therefore, hypoxia might play a role during regeneration process, while FAPs communicate with satellite cells. Thus, we asked if hypoxia would influence the cross talk between these two progenitor populations. FAPs were amplified and the EV-depleted FAPs CM was produced under normoxic and hypoxic conditions. We observed that EV-depleted FAPs CM led to the enrichment of Pax7+/MyoD- cells independently of the oxygen level used during its conditioning (Figure 29). Although it has been previously suggested a potential direct role of hypoxia on satellite cells, favoring satellite cell self renewal (Liu et al., 2012), when the EV-depleted FAPs CM is conditioned under hypoxic environment neither disturbed nor improved the satellite cell self-renewal (Figure 29).

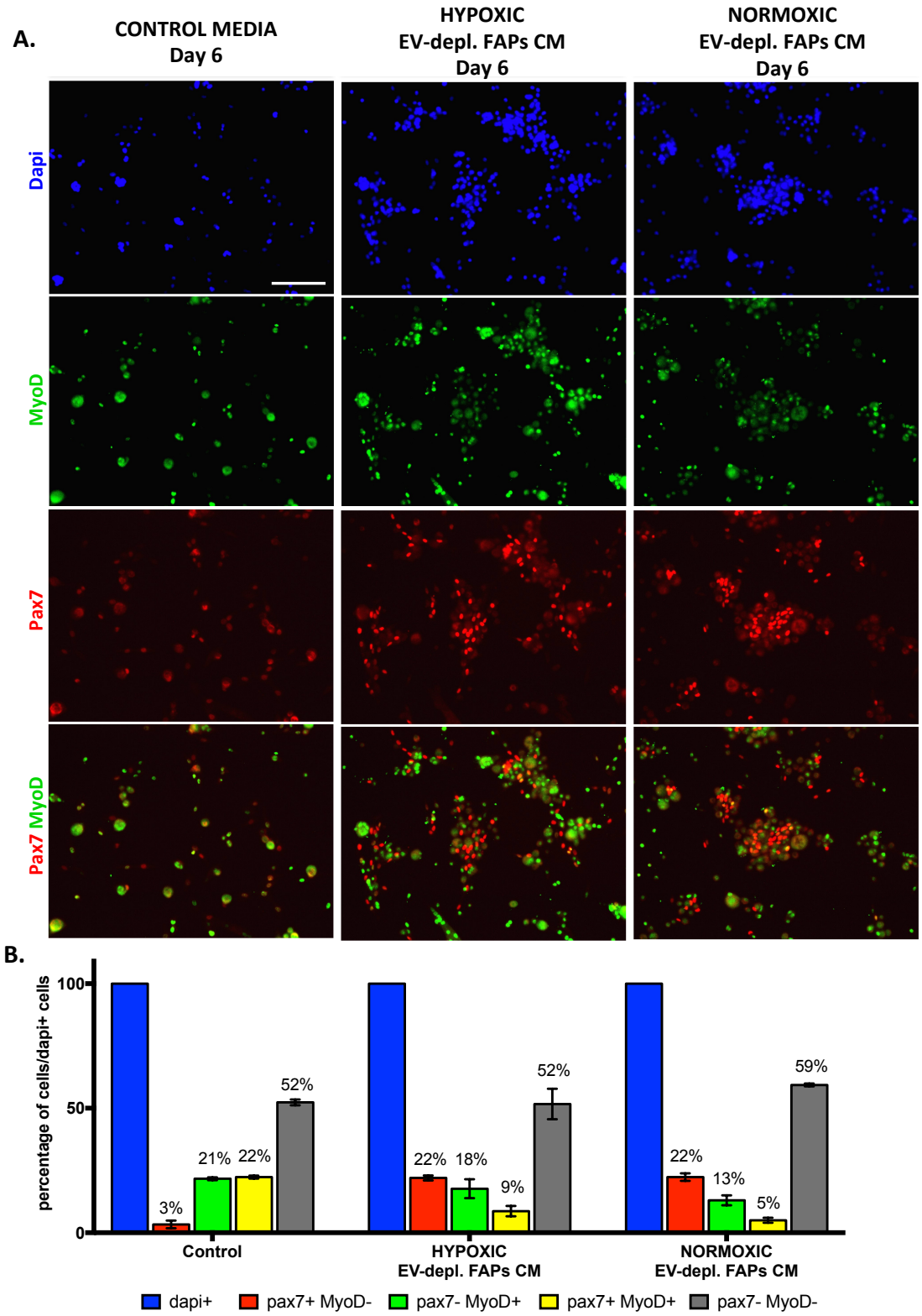

Figure 29. Hypoxia does not influence the number of Pax7+/MyoD- cells. Satellite cells after three days in amplification media, were incubated for three more days with EV-depleted FAPs CM produced culturing FAPs in normoxic or hypoxic condition, or un-conditioned media as control. (A) Staining of satellite cells at day 6 for Pax7 (red), MyoD (green); scale bar 100µm. (B) Quantification of the percentage of cells on total cell number of dapi+ nuclei.

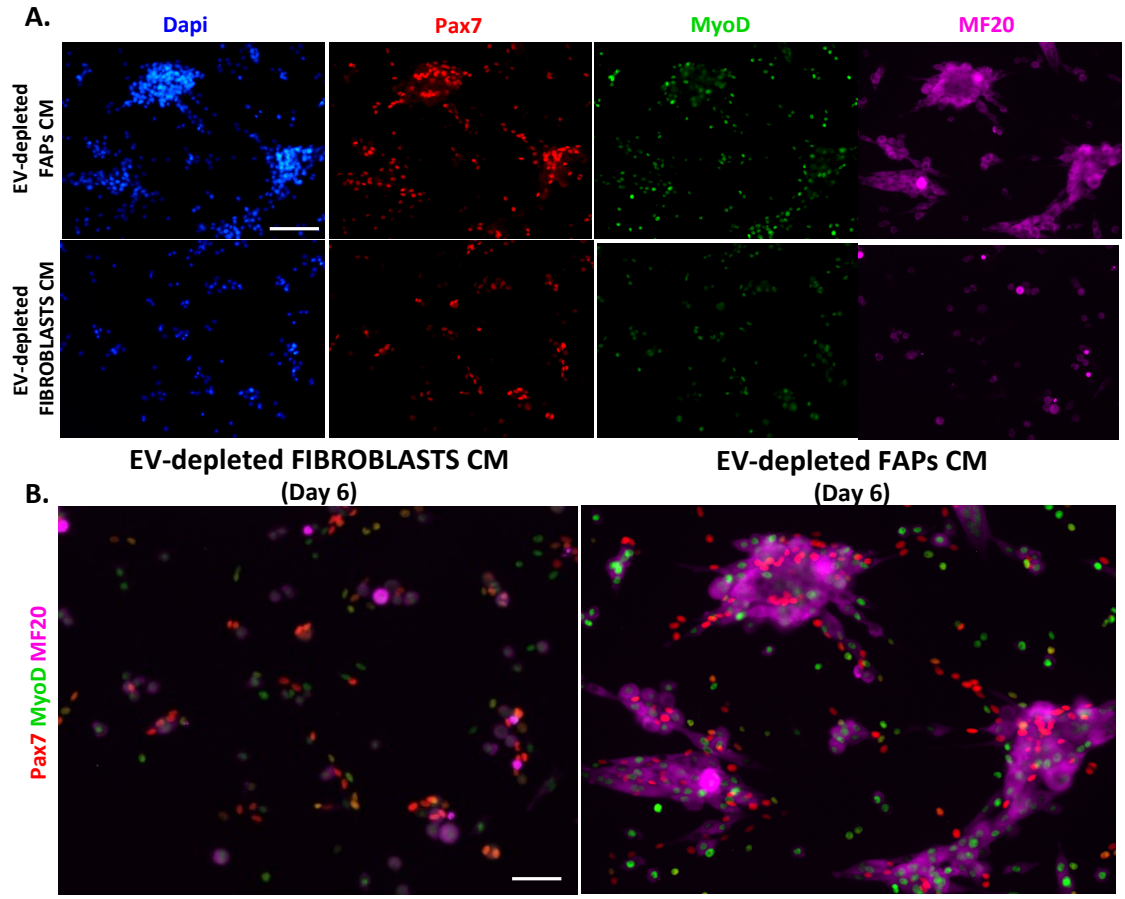

Figure 30. The effect of EV-depleted FAPs conditioned media is mediated by factors released by specifically by FAPs. Satellite cells were incubated with EV-depleted FAPs conditioned media or EV-depleted fibroblasts conditioned media for three days after three days of amplification in culture. (A) Staining of satellite cells for Pax7 (red) and MyoD (green) and MF20 (magenta); scale bar 100μm. (B) Higher magnification of the merge; scale bar 50μm.

The Pax7+MyoD- satellite cells enrichment within grapes is mediated by factors specifically released by FAPs

We next asked if the effect of satellite cells growing in grapes is specifically related to FAPs. In order to check this possibility we prepared conditioned media from mouse skin fibroblasts and observed that satellite cells cultured with fibroblast-conditioned medium did not acquire the aciniform morphology (Figure 30). In line with this observation, also the number of Pax7+/MyoD- cells was lower when satellite cells are cultured with fibroblast-CM compared to FAP-CM cultured satellite cells (Figure 30). This result let us to conclude that the observed effect is FAP-related and not merely

due to spurious products of “exhausted” media from cells during the media conditioning. The FAP-CM contains the key regulator/s responsible for the induction of the Pax7+/MyoD- satellite cell phenotype.

NOGGIN suppresses the satellite cells self-renewal mediated by FAPs

Identifying the specific factors within a medium enriched in all the FAPs secreted molecules can be a laborious and endless process. We thus decided to investigate the RNA profile expression, narrowing our potential targets by selecting FAPs secreted molecules known to be involved in activation, proliferation and self-renewal of satellite cells, looking at RNA profile expression.

Notch pathway has been reported as a key regulator of the satellite cell quiescence (Mourikis et al., 2012) (Almada and Wagers, 2016), where the downstream effectors downregulate MyoD and induce Pax7 expressions (Almada and Wagers, 2016). Although the canonical ligands of Notch receptors are membrane associated proteins, thus involving juxtaposed cells in the communication and the consequent activation and signal transduction, there are some non-canonical ligands, including MFAP proteins, thrombospondin 2 and CCN3 that act as secreted factors (D'Souza et al., 2010). In our analyses, we found CCN3/NOV, MFAP5 and MFAP2 to be expressed at a higher level in FAPs, compared to satellite cells (Figure 31b). Fibroblast growth factors (FGFs) signaling is also involved in satellite cells activation as well as in regulation of self-renewal (Pawlikowski et al., 2017) (Hashimoto et al., 2004). We found some members of this pathway, such as FGF-1, FGF7 and FGF-10, being highly expressed by FAPs (Figure 31c). Finally, bone morphogenetic proteins (BMPs), a subgroup of the transforming growth factor (TGF)-beta super family, are commonly considered strong inhibitors of muscle differentiation (Dahlqvist et al., 2003) and to function in satellite cells proliferation (Ono et al., 2011). We also observed some of this family members highly enriched in FAPs as compared to satellite cells (Figure 31a).

In light of these data, we focused on BMP signaling, evaluating the effect of EV-depleted FAPs CM supplemented with the BMP signaling antagonist Noggin. The latter is a secreted protein that, by binding non-covalently BMPs, is able to impede the interaction with receptors (Krause et al., 2011). The presence of Noggin in the EV-depleted FAPs CM, led to a significant decrease of grape formation as compared to the EV-depleted FAPs CM alone (in the absence of Noggin). The decrease of the satellite

cell grapes is paralleled by decrease on the Pax7+/MyoD- cells from almost 40% to 14% (Figure 32). We also observed a slight decrease (from 29% to 22%) in the myoblast-committed population (Pax7-MyoD+) and an increase in negative population, when Noggin is added to the EV-depleted FAPs CM. In line with this finding, it has been reported that endogenous Noggin in satellite cells inhibits BMP proteins to assist myogenic differentiation (Ono et al., 2011). Furthermore, Bmp4 is released by tendons, influencing a subpopulation of Pax7+ cells located at the tips of muscle by stimulating progenitors proliferation (Wang et al., 2010); the exogenous BMP4 is also been reported to promote satellite cell division (Ono et al., 2011).

Overall, our results suggested a potential involvement of BMP proteins in the induction of satellite cell self-renewal mediated by EV-depleted FAPs CM, being thus good candidates of further studies.

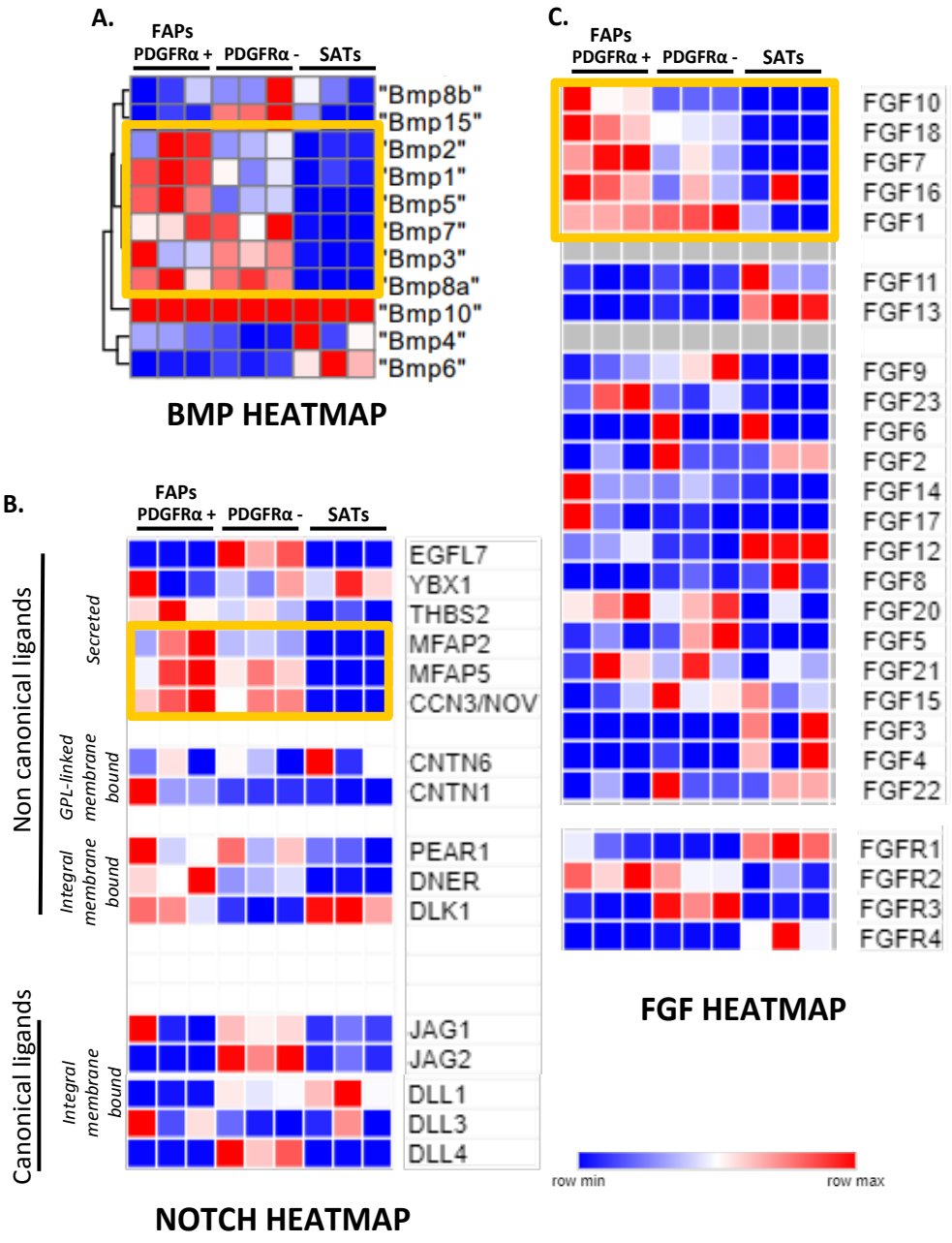

Figure 31. Secreted molecules enriched in FAPs might be involved in self-renewal. Heatmaps representing the RNA expression profile of factors involved in self renewal/proliferation of satellite cells, as BMPs family (A), Notch ligands (B), and FGF family (C).

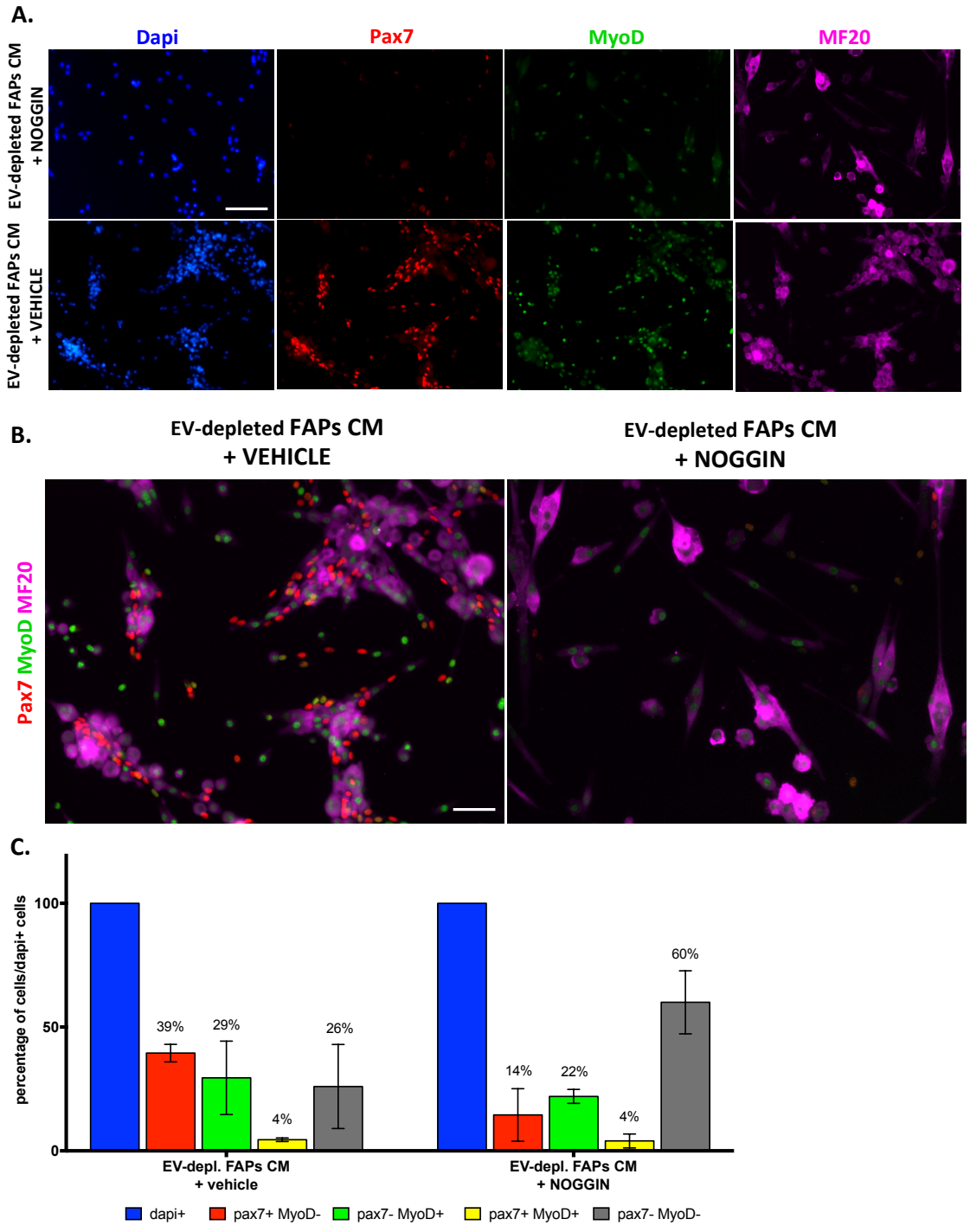

Figure 32. NOGGIN suppressed the self renewing like phenotype mediated by EV-depleted FAPs CM. Satellite cells were amplified three days in culture, then incubated with EV-depleted FAPs CM with the addition of Noggin. (A) Staining of satellite cells for Pax7 (red) and MyoD (green) and MF20 (magenta); scale bar 100µm. (B) Higher magnification of the merge; scale bar 50µm. (C) Quantification of the percentage of cells on total cell number of dapi+ nuclei.

CONCLUSION

The ability of satellite cells to maintain the pool of quiescent cells is fundamental to ensure the source of stem cells to regenerate muscle damaged after every injury or exercise. This capacity is depending on the muscle stem cell niche, the crucial microenvironment surrounding satellite cells. Indeed, it is known that when isolated from the niche, satellite cells lose their self-renewal potential; this event represents a huge limitation for regenerative medical applications, where the ex vivo step of satellite cells culture/expansion is required as well as the preservation of their regenerative potential.

Here, we investigated the influence of tissue-resident mesenchymal stromal cells (the fibro-adipogenic progenitors) on satellite cell self-renewal. We looked at the impact of FAPs conditioned media on satellite cell culture. We found that FAPs specific soluble factors present in the conditioned media promote satellite cell proliferation and enhance the number of Pax7+ MyoD- satellite cells. Our satellite cell culture conditions, similarly to the stem cell niche environment allow to keep satellite cells ability to self renew and adopt a divergent fate after an activation phase. Our evidences let us propose FAPs as key actors on regulation of satellite cell fate decision through their paracrine signaling, and suggest new insight to unravel the molecular mechanism involved in satellite cell self-renewal.

REFERENCES

Please see references at *REFERENCES*.

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Besides the muscle fibers and their associated myogenic satellite cells, tissue-resident mesenchymal stromal cells, vascular and immune system cells are effective part of the skeletal muscle stem cell niche. The intercellular communication between all the cellular components within the stem cell niche is crucial during development, homeostasis and regenerative process. My thesis focuses on these intercellular communications, the cellular players and the molecular regulation.

Cell-to-cell communication can be direct, occurring through contact of the two cell types, as it is the case of satellite cells and the myofiber; or indirect, mediated by the signals transfer by extracellular vesicles (EVs) or soluble factors (Dumont et al., 2015).

Satellite cells can release Alix-positive exosomes directly from their plasma membranes, being Alix determinant in this process (Romancino et al., 2013). In the context of intercellular communication mediated by EVs, the first chapter of this thesis describes the mechanisms regulating the skeletal muscle cell-derived EV biogenesis and cargo loading. In particular, we focused in more detail at Alix function, a *bona fide* exosomal regulator, and its regulation by palmitoylation using a murine myogenic cell line – C2C12 cells. Protein S-palmitoylation is a reversible post-translational (PTM) lipid modification that is involved in different biological processes (Aicart-Ramos et al., 2011), however its specific role is still poorly understood. In the present work, we asked if the dynamic features of S-palmitoylation are implicated in the regulation of orderly EV production and organization in skeletal muscle cells. Using a bioinformatics approach we found that palmitoylated proteins are enriched in exosomes, indicating this PTM as a good candidate for a specific signature of exosomal proteins. In line with this finding, in vitro data showed that a prenylation/palmitoylation tag can target a recombinant, highly oligomeric cytoplasmic protein into exosomes (Shen et al., 2011). Thus, we have applied an integrated biochemical-biophysical approach to examine whether S-palmitoylation modulates Alix function and maintains the integrity of exosomal membranes. Indeed, we confirmed the presence of S-palmitoylated Alix pool in skeletal muscle cell-derived exosomes. Our study also unveils an unsuspected association of Alix with a member of the tetraspanin protein family, the CD9, in the context of the

tetraspanin-enriched microdomains (TEM) of myogenic C2C12 cell membrane. We perturbed the palmitoylation of Alix, by using the specific inhibitor (2BP), and we observed that the interaction between Alix and CD9 was altered. Moreover, we also detected the production of different nano-sized vesicles that differ in terms of protein cargo and membrane structure. These results are consistent with previous reports showing that cells secrete exosome populations heterogeneous in size and composition (Bobrie et al., 2012) (van Niel et al., 2001) (Colombo et al., 2013). We, thus hypothesize that the equilibrium between the different subpopulation of nano-sized vesicles produced by the myogenic C2C12 cells is deregulated. It is then possible that other exosome subpopulations are released to compensate the deregulation of the palmitoyl-related exosomal pool. Altogether, our study suggests that S-palmitoylation influences the interaction between Alix and CD9 and maintains the proper organization of exosome membrane structure, in addition to regulating the proper function of membrane-associated Alix in exosome biogenesis. Considering the role of tetraspanin microdomains (TEM, of which CD9 is part) in exosomes biogenesis, the importance of S-palmitoylation in the tetraspanin microdomains organization and our own results, it is possible postulate that the interaction between the palmitoylated Alix with the cell membrane TEM happens through CD9 contact. Alix–CD9 interaction is sensitive to the presence of the PTM S-palmitoylation, and consequently impacts the production of Alix positive exosome population (Figure 33).
Understanding the mechanisms of protein loading into exosomes and the role of post-translational modifications will enable to develop strategies to target specific proteins in exosomes for possible therapeutic approaches.

Chapter II of this thesis focus on another type of cell communication that also occurs in muscle stem cell niche, which is carried out by paracrine factors released by fibro-adipogenic progenitors (FAPs). FAPs are known to be important during muscle regeneration, promoting myogenesis and supporting new muscle formation with the release of extracellular matrix (Joe et al., 2010) (Natarajan et al., 2010). Our work shows that FAPs-derived soluble factors are able to promote primary satellite cells proliferation in vitro, maintaining their primitive stem-like (Pax7+/MyoD-) identity. These stem-like (Pax7+/MyoD-) satellite cells grow in 3D and in grape like structure (i.e. aciniform structure) and always on top of myotubes, demonstrating the importance of the direct contact between the two cell types for the maintenance of stem-like identity of satellite cells, as it happens in vivo for quiescent satellite cells (Goel et al., 2017). We are thus showing that beyond the already known fibrotic and adipogenic (Uezumi et al., 2010) (Natarajan et al., 2010) and endothelial (Ollitrault D, 2017) potentials,

FAPs also produce paracrine factors that induce the expansion of satellite cells with self-renewal phenotype. Thus, we analyzed the expression of soluble factors correlated with satellite cell self-renewal, and inhibited some of them (using Noggin) to identify their functional role. We could indicate members of the BMPs family as the signaling molecules released by FAPs to induce self-renewal and differentiation of satellite cells (Figure 34).

It is remarkable the ability to expand Pax7+/MyoD- satellite cells in vitro, which is a gold standard that has been followed during the last years without success, because of the sensitivity of this stem cell state to microenvironment change. The capacity of a stem cell to self-renew and differentiate has been associated to the ability to undergo asymmetric division, besides classical symmetrical division and differentiation process. The asymmetric division ensures the differentiation and the replenishment of the stem cell pool in the same time. What we reproduced in vitro instead, it is a mechanism that is not following the classical symmetrical and asymmetrical roads, but a more plastic and reversible behavior where satellite cells acquire a self-renewal phenotype passing through an intermediate committed state (Pax7+, MyoD+).

Although this work is still ongoing, it is clear the potential milestone impact in the muscle field of preserving the satellite cell stemness in vitro, which can lead to the discovery of molecules for new technology development and new lines of research.

Taken together all the work presented in this thesis, we were able to explore multiple faces of the skeletal muscle niche communication, which opened new important lines of research.

In light of the Chapter I results, it would be interesting to continue the research and deepen some more aspects in the study on EV-derived from myogenic cells and their regulation mediated by S-palmitoylation. The next step would be analyzing the EV population when Alix is mutated in the predicted palmitoylation site. This would give us the confirmation of the site of palmitoylation, but also the possibility to study the palmitoylation effect specifically on Alix. Furthermore, the role of Alix in the biogenesis and the heterogeneity of myogenic cells-derived exosomes will be further confirmed and addressed by characterization of the Alix animal models (generated in Dr. d'Azzo lab at St. Jude Children Research Hospital, Memphis-USA) (Campos et al., 2016).

Regarding the FAP-secreted soluble signal/s, findings encouraged us to further characterize the enriched cell population (Pax7+/MyoD-), its proliferative capability and expression profile; for example, a transgenic mouse strain expressing a fluorescent protein under the promoter of a gene of interest, as MyoD, would be useful to follow the

satellite cell fate, either in vitro or in vivo. The final goal would be the identification of the molecules or the cocktail of soluble factors that trigger satellite cell self-renewal as well as the digging of the pathway involved in the satellite cell self-renewal. For this aim, applying a gene editing system to modified FAPs, would give as the confirmation of the secreted molecule acting on satellite cells.

Additionally, considering the FAP influence on the promotion of satellite cell self-renewal, it would be important to explore the potential contribution of the metabolic composition of the media. Indeed, satellite cells change in size and metabolic activity when they transit from a quiescent to a proliferating state (Koopman et al., 2014). Thus, it might exist a link between metabolic sensing and satellite cell quiescence and activation. In line with this suggestion, calorie-restricted mice are characterized for their enhanced muscle regenerative activity and satellite cells have been found to have increased oxygen consumption and mitochondrial activity in these mice (Cerletti et al., 2012). Thus, it would be interesting to analyze the potential synergic role of metabolic components and the FAP-secreted signals on the satellite cell fate decision.

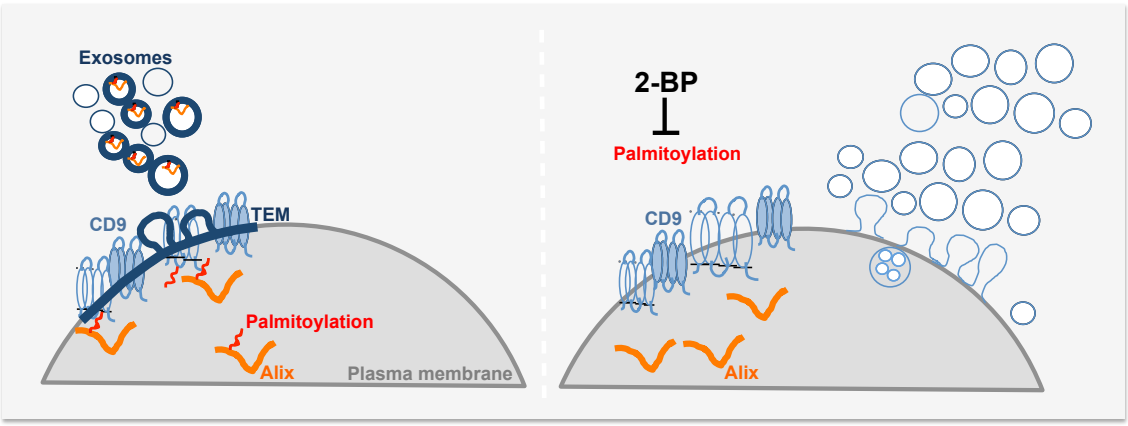

Figure 33. Palmitoylation is a post-translational modification of Alix regulating the membrane organization of exosomes. In skeletal muscle cell (i.e. C2C12), the S-palmitoylation is a key post-translational modification of the *bona fide* exosome regulator Alix, allowing its interaction with CD9, a member of the tetraspanin enriched microdomains. Skeletal muscle-derived exosomes are enriched in palmitoyl-Alix. When palmitoylation is inhibited by 2BP, the interaction between Alix and CD9 is altered, leading to a deregulation of exosome formation and membrane organization.

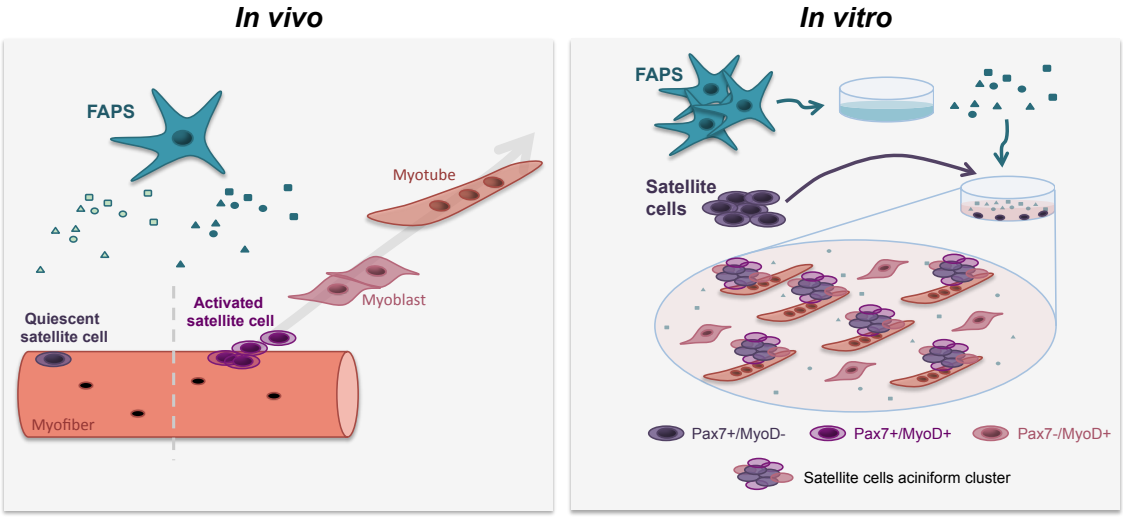

Figure 34. Regulation of satellite cell self-renewal mediated by FAPs. In vivo, in the muscle stem niche, quiescent satellite cells (Pax7+/MyoD-) reside on the myofiber beside the basal lamina. All the other components of the niche take part to maintain the satellite cell competence and function. FAPs interact with satellite cells acting in a paracrine fashion. In our model in vitro, FAPs secreted factors act on satellite cells, promoting satellite cells growth with aciniform morphology on the top of small myotubes, leading to the enrichment of Pax7+/MyoD- cells. BMPs are the candidate molecules released by FAPs and able to influence self-renewal of satellite cells.

REFERENCES

Abou-Khalil, R., Le Grand, F., Pallafacchina, G., Valable, S., Authier, F.J., Rudnicki, M.A., Gherardi, R.K., Germain, S., Chretien, F., Sotiropoulos, A.*, et al.* (2009). Autocrine and paracrine angiopoietin 1/Tie-2 signaling promotes muscle satellite cell self-renewal. Cell Stem Cell *5*, 298-309.

Aicart-Ramos, C., Valero, R.A., and Rodriguez-Crespo, I. (2011). Protein palmitoylation and subcellular trafficking. Biochim Biophys Acta *1808*, 2981-2994.

Al-Nedawi, K., Meehan, B., Kerbel, R.S., Allison, A.C., and Rak, J. (2009). Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. Proc Natl Acad Sci U S A *106*, 3794-3799.

Alberts B, J.A., Lewis J, et al. (2002). Molecular Biology of the Cell, 4th edition.

Allen, D.L., Hittel, D.S., and McPherron, A.C. (2011). Expression and function of myostatin in obesity, diabetes, and exercise adaptation. Med Sci Sports Exerc *43*, 1828-1835.

Almada, A.E., and Wagers, A.J. (2016). Molecular circuitry of stem cell fate in skeletal muscle regeneration, ageing and disease. Nat Rev Mol Cell Biol *17*, 267-279.

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M.J. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol *29*, 341-345.

Andaloussi, S.E.L., Mager, I., Breakefield, X.O., and Wood, M.J. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov *12*, 347-357.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166-169.

Argiles, J.M., Lopez-Soriano, J., Almendro, V., Busquets, S., and Lopez-Soriano, F.J. (2005). Cross-talk between skeletal muscle and adipose tissue: a link with obesity? Med Res Rev *25*, 49-65.

Ashburner, M., Ball, C.A., Blake, J.A., Botstein, D., Butler, H., Cherry, J.M., Davis, A.P., Dolinski, K., Dwight, S.S., Eppig, J.T.*, et al.* (2000). Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet *25*, 25-29.

Baar, K., Wende, A.R., Jones, T.E., Marison, M., Nolte, L.A., Chen, M., Kelly, D.P., and Holloszy, J.O. (2002). Adaptations of skeletal muscle to exercise: rapid increase in the transcriptional coactivator PGC-1. FASEB J *16*, 1879-1886.

Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E.*, et al.* (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol *14*, 677-685.

Beauchamp, J.R., Heslop, L., Yu, D.S., Tajbakhsh, S., Kelly, R.G., Wernig, A., Buckingham, M.E., Partridge, T.A., and Zammit, P.S. (2000). Expression of CD34 and

Myf5 defines the majority of quiescent adult skeletal muscle satellite cells. J Cell Biol *151*, 1221-1234.

Bentzinger, C.F., Wang, Y.X., Dumont, N.A., and Rudnicki, M.A. (2013). Cellular dynamics in the muscle satellite cell niche. EMBO Rep *14*, 1062-1072.

Bentzinger, C.F., Wang, Y.X., and Rudnicki, M.A. (2012). Building muscle: molecular regulation of myogenesis. Cold Spring Harb Perspect Biol *4*.

Besson, V., Smeriglio, P., Wegener, A., Relaix, F., Nait Oumesmar, B., Sassoon, D.A., and Marazzi, G. (2011). PW1 gene/paternally expressed gene 3 (PW1/Peg3) identifies multiple adult stem and progenitor cell populations. Proc Natl Acad Sci U S A *108*, 11470-11475.

Bianco, F., Perrotta, C., Novellino, L., Francolini, M., Riganti, L., Menna, E., Saglietti, L., Schuchman, E.H., Furlan, R., Clementi, E.*, et al.* (2009). Acid sphingomyelinase activity triggers microparticle release from glial cells. EMBO J *28*, 1043-1054.

Bischoff, R. (1986). Proliferation of muscle satellite cells on intact myofibers in culture. Dev Biol *115*, 129-139.

Bismuth, K., and Relaix, F. (2010). Genetic regulation of skeletal muscle development. Exp Cell Res *316*, 3081-3086.

Bissig, C., and Gruenberg, J. (2014). ALIX and the multivesicular endosome: ALIX in Wonderland. Trends Cell Biol *24*, 19-25.

Bissig, C., Lenoir, M., Velluz, M.C., Kufareva, I., Abagyan, R., Overduin, M., and Gruenberg, J. (2013). Viral infection controlled by a calcium-dependent lipid-binding module in ALIX. Dev Cell *25*, 364-373.

Bjornson, C.R., Cheung, T.H., Liu, L., Tripathi, P.V., Steeper, K.M., and Rando, T.A. (2012). Notch signaling is necessary to maintain quiescence in adult muscle stem cells. Stem Cells *30*, 232-242.

Blanc, M., David, F., Abrami, L., Migliozzi, D., Armand, F., Burgi, J., and van der Goot, F.G. (2015). SwissPalm: Protein Palmitoylation database. F1000Res *4*, 261.

Blanco-Bose, W.E., Yao, C.C., Kramer, R.H., and Blau, H.M. (2001). Purification of mouse primary myoblasts based on alpha 7 integrin expression. Exp Cell Res *265*, 212-220.

Blaskovic, S., Blanc, M., and van der Goot, F.G. (2013). What does S-palmitoylation do to membrane proteins? FEBS J *280*, 2766-2774.

Blau, H.M., Pavlath, G.K., Hardeman, E.C., Chiu, C.P., Silberstein, L., Webster, S.G., Miller, S.C., and Webster, C. (1985). Plasticity of the differentiated state. Science *230*, 758-766.

Blum, D., Hemming, F.J., Galas, M.C., Torch, S., Cuvelier, L., Schiffmann, S.N., and Sadoul, R. (2004). Increased Alix (apoptosis-linked gene-2 interacting protein X)

immunoreactivity in the degenerating striatum of rats chronically treated by 3-nitropropionic acid. Neurosci Lett *368*, 309-313.

Bobrie, A., Colombo, M., Krumeich, S., Raposo, G., and Thery, C. (2012). Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation. J Extracell Vesicles *1*.

Bongiovanni, A., Romancino, D.P., Campos, Y., Paterniti, G., Qiu, X., Moshiach, S., Di Felice, V., Vergani, N., Ustek, D., and d'Azzo, A. (2012). Alix protein is substrate of Ozz-E3 ligase and modulates actin remodeling in skeletal muscle. J Biol Chem *287*, 12159-12171.

Booth, A.M., Fang, Y., Fallon, J.K., Yang, J.M., Hildreth, J.E., and Gould, S.J. (2006). Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. J Cell Biol *172*, 923-935.

Bosurgi, L., Corna, G., Vezzoli, M., Touvier, T., Cossu, G., Manfredi, A.A., Brunelli, S., and Rovere-Querini, P. (2012). Transplanted mesoangioblasts require macrophage IL-10 for survival in a mouse model of muscle injury. J Immunol *188*, 6267-6277.

Boukouris, S., and Mathivanan, S. (2015). Exosomes in bodily fluids are a highly stable resource of disease biomarkers. Proteomics Clin Appl *9*, 358-367.

Bouwstra, J.A., Gooris, G.S., Bras, W., and Talsma, H. (1993). Small angle X-ray scattering: possibilities and limitations in characterization of vesicles. Chem Phys Lipids *64*, 83-98.

Boyd, A.E., 3rd, Giamber, S.R., Mager, M., and Lebovitz, H.E. (1974). Lactate inhibition of lipolysis in exercising man. Metabolism *23*, 531-542.

Brigidi, G.S., and Bamji, S.X. (2013). Detection of protein palmitoylation in cultured hippocampal neurons by immunoprecipitation and acyl-biotin exchange (ABE). J Vis Exp.

Brohl, D., Vasyutina, E., Czajkowski, M.T., Griger, J., Rassek, C., Rahn, H.P., Purfurst, B., Wende, H., and Birchmeier, C. (2012). Colonization of the satellite cell niche by skeletal muscle progenitor cells depends on Notch signals. Dev Cell *23*, 469-481.

Broholm, C., Laye, M.J., Brandt, C., Vadalasetty, R., Pilegaard, H., Pedersen, B.K., and Scheele, C. (2011). LIF is a contraction-induced myokine stimulating human myocyte proliferation. J Appl Physiol (1985) *111*, 251-259.

Buckingham, M., and Montarras, D. (2008). Skeletal muscle stem cells. Curr Opin Genet Dev *18*, 330-336.

Burke, M.C., Oei, M.S., Edwards, N.J., Ostrand-Rosenberg, S., and Fenselau, C. (2014). Ubiquitinated proteins in exosomes secreted by myeloid-derived suppressor cells. J Proteome Res *13*, 5965-5972.

Cai, Z., Yang, F., Yu, L., Yu, Z., Jiang, L., Wang, Q., Yang, Y., Wang, L., Cao, X., and Wang, J. (2012). Activated T cell exosomes promote tumor invasion via Fas signaling pathway. J Immunol *188*, 5954-5961.

Campos, Y., Qiu, X., Gomero, E., Wakefield, R., Horner, L., Brutkowski, W., Han, Y.G., Solecki, D., Frase, S., Bongiovanni, A.*, et al.* (2016). Alix-mediated assembly of the actomyosin-tight junction polarity complex preserves epithelial polarity and epithelial barrier. Nat Commun *7*, 11876.

Carlton, J.G., Agromayor, M., and Martin-Serrano, J. (2008). Differential requirements for Alix and ESCRT-III in cytokinesis and HIV-1 release. Proc Natl Acad Sci U S A *105*, 10541-10546.

Castorph, S., Riedel, D., Arleth, L., Sztucki, M., Jahn, R., Holt, M., and Salditt, T. (2010). Structure parameters of synaptic vesicles quantified by small-angle x-ray scattering. Biophys J *98*, 1200-1208.

Cerletti, M., Jang, Y.C., Finley, L.W., Haigis, M.C., and Wagers, A.J. (2012). Short-term calorie restriction enhances skeletal muscle stem cell function. Cell Stem Cell *10*, 515-519.

Chairoungdua, A., Smith, D.L., Pochard, P., Hull, M., and Caplan, M.J. (2010). Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling. J Cell Biol *190*, 1079-1091.

Chang, A.J., Ortega, F.E., Riegler, J., Madison, D.V., and Krasnow, M.A. (2015). Oxygen regulation of breathing through an olfactory receptor activated by lactate. Nature *527*, 240-244.

Charge, S.B., and Rudnicki, M.A. (2004). Cellular and molecular regulation of muscle regeneration. Physiol Rev *84*, 209-238.

Charrin, S., Jouannet, S., Boucheix, C., and Rubinstein, E. (2014). Tetraspanins at a glance. J Cell Sci *127*, 3641-3648.

Charrin, S., Latil, M., Soave, S., Polesskaya, A., Chretien, F., Boucheix, C., and Rubinstein, E. (2013). Normal muscle regeneration requires tight control of muscle cell fusion by tetraspanins CD9 and CD81. Nat Commun *4*, 1674.

Charrin, S., Manie, S., Billard, M., Ashman, L., Gerlier, D., Boucheix, C., and Rubinstein, E. (2003). Multiple levels of interactions within the tetraspanin web. Biochem Biophys Res Commun *304*, 107-112.

Charrin, S., Manie, S., Oualid, M., Billard, M., Boucheix, C., and Rubinstein, E. (2002). Differential stability of tetraspanin/tetraspanin interactions: role of palmitoylation. FEBS Lett *516*, 139-144.

Chen, C., and Sytkowski, A.J. (2005). Apoptosis-linked gene-2 connects the Raf-1 and ASK1 signalings. Biochem Biophys Res Commun *333*, 51-57.

Chen, X., Jayne Lawrence, M., Barlow, D.J., Morris, R.J., Heenan, R.K., and Quinn, P.J. (2009). The structure of detergent-resistant membrane vesicles from rat brain cells. Biochim Biophys Acta *1788*, 477-483.

Chesarino, N.M., Hach, J.C., Chen, J.L., Zaro, B.W., Rajaram, M.V., Turner, J., Schlesinger, L.S., Pratt, M.R., Hang, H.C., and Yount, J.S. (2014). Chemoproteomics reveals Toll-like receptor fatty acylation. BMC Biol *12*, 91.

Cheung, K.H., Keerthikumar, S., Roncaglia, P., Subramanian, S.L., Roth, M.E., Samuel, M., Anand, S., Gangoda, L., Gould, S., Alexander, R.*, et al.* (2016). Extending gene ontology in the context of extracellular RNA and vesicle communication. J Biomed Semantics *7*, 19.

Chiavegatti, T., Costa, V.L., Jr., Araujo, M.S., and Godinho, R.O. (2008). Skeletal muscle expresses the extracellular cyclic AMP-adenosine pathway. Br J Pharmacol *153*, 1331-1340.

Choi, J.S., Yoon, H.I., Lee, K.S., Choi, Y.C., Yang, S.H., Kim, I.S., and Cho, Y.W. (2016). Exosomes from differentiating human skeletal muscle cells trigger myogenesis of stem cells and provide biochemical cues for skeletal muscle regeneration. J Control Release *222*, 107-115.

Christov, C., Chretien, F., Abou-Khalil, R., Bassez, G., Vallet, G., Authier, F.J., Bassaglia, Y., Shinin, V., Tajbakhsh, S., Chazaud, B.*, et al.* (2007). Muscle satellite cells and endothelial cells: close neighbors and privileged partners. Mol Biol Cell *18*, 1397-1409.

Ciardiello, C., Cavallini, L., Spinelli, C., Yang, J., Reis-Sobreiro, M., de Candia, P., Minciacchi, V.R., and Di Vizio, D. (2016). Focus on Extracellular Vesicles: New Frontiers of Cell-to-Cell Communication in Cancer. Int J Mol Sci *17*, 175.

Cocucci, E., and Meldolesi, J. (2015). Ectosomes and exosomes: shedding the confusion between extracellular vesicles. Trends Cell Biol *25*, 364-372.

Cocucci, E., Racchetti, G., and Meldolesi, J. (2009). Shedding microvesicles: artefacts no more. Trends Cell Biol *19*, 43-51.

Cohn, R.D., Henry, M.D., Michele, D.E., Barresi, R., Saito, F., Moore, S.A., Flanagan, J.D., Skwarchuk, M.W., Robbins, M.E., Mendell, J.R.*, et al.* (2002). Disruption of DAG1 in differentiated skeletal muscle reveals a role for dystroglycan in muscle regeneration. Cell *110*, 639-648.

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L.F., Thery, C., and Raposo, G. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci *126*, 5553-5565.

Colombo, M., Raposo, G., and Thery, C. (2014). Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. Annu Rev Cell Dev Biol *30*, 255-289.

Cordani, N., Pisa, V., Pozzi, L., Sciorati, C., and Clementi, E. (2014). Nitric oxide controls fat deposition in dystrophic skeletal muscle by regulating fibro-adipogenic precursor differentiation. Stem Cells *32*, 874-885.

D'Souza, B., Meloty-Kapella, L., and Weinmaster, G. (2010). Canonical and non-canonical Notch ligands. Curr Top Dev Biol *92*, 73-129.

Dahlqvist, C., Blokzijl, A., Chapman, G., Falk, A., Dannaeus, K., Ibanez, C.F., and Lendahl, U. (2003). Functional Notch signaling is required for BMP4-induced inhibition of myogenic differentiation. Development *130*, 6089-6099.

Del Conde, I., Shrimpton, C.N., Thiagarajan, P., and Lopez, J.A. (2005). Tissue-factor-bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation. Blood *106*, 1604-1611.

Deng, B., Wehling-Henricks, M., Villalta, S.A., Wang, Y., and Tidball, J.G. (2012). IL-10 triggers changes in macrophage phenotype that promote muscle growth and regeneration. J Immunol *189*, 3669-3680.

Dores, M.R., Lin, H., N, J.G., Mendez, F., and Trejo, J. (2015). The alpha-arrestin ARRDC3 mediates ALIX ubiquitination and G protein-coupled receptor lysosomal sorting. Mol Biol Cell *26*, 4660-4673.

Dumont, N.A., Wang, Y.X., and Rudnicki, M.A. (2015). Intrinsic and extrinsic mechanisms regulating satellite cell function. Development *142*, 1572-1581.

Elhelu, M.A. (1983). The role of macrophages in immunology. J Natl Med Assoc *75*, 314-317.

Escrevente, C., Keller, S., Altevogt, P., and Costa, J. (2011). Interaction and uptake of exosomes by ovarian cancer cells. BMC Cancer *11*, 108.

Fang, Y., Wu, N., Gan, X., Yan, W., Morrell, J.C., and Gould, S.J. (2007). Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. PLoS Biol *5*, e158.

Fenton, R.A., Moeller, H.B., Hoffert, J.D., Yu, M.J., Nielsen, S., and Knepper, M.A. (2008). Acute regulation of aquaporin-2 phosphorylation at Ser-264 by vasopressin. Proc Natl Acad Sci U S A *105*, 3134-3139.

Fiore, D., Judson, R.N., Low, M., Lee, S., Zhang, E., Hopkins, C., Xu, P., Lenzi, A., Rossi, F.M., and Lemos, D.R. (2016). Pharmacological blockage of fibro/adipogenic progenitor expansion and suppression of regenerative fibrogenesis is associated with impaired skeletal muscle regeneration. Stem Cell Res *17*, 161-169.

Fisher, R.D., Chung, H.Y., Zhai, Q., Robinson, H., Sundquist, W.I., and Hill, C.P. (2007). Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. Cell *128*, 841-852.

Fry, C.S., Kirby, T.J., Kosmac, K., McCarthy, J.J., and Peterson, C.A. (2017). Myogenic Progenitor Cells Control Extracellular Matrix Production by Fibroblasts during Skeletal Muscle Hypertrophy. Cell Stem Cell *20*, 56-69.

Fukata, Y., and Fukata, M. (2010). Protein palmitoylation in neuronal development and synaptic plasticity. Nat Rev Neurosci *11*, 161-175.

Furmanczyk, P.S., and Quinn, L.S. (2003). Interleukin-15 increases myosin accretion in human skeletal myogenic cultures. Cell Biol Int *27*, 845-851.

Gardiner, C., Di Vizio, D., Sahoo, S., Thery, C., Witwer, K.W., Wauben, M., and Hill, A.F. (2016). Techniques used for the isolation and characterization of extracellular vesicles: results of a worldwide survey. J Extracell Vesicles *5*, 32945.

Gatti, S., Bruno, S., Deregibus, M.C., Sordi, A., Cantaluppi, V., Tetta, C., and Camussi, G. (2011). Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia-reperfusion-induced acute and chronic kidney injury. Nephrol Dial Transplant *26*, 1474-1483.

Gene Ontology, C. (2006). The Gene Ontology (GO) project in 2006. Nucleic Acids Res *34*, D322-326.

Gilbert, P.M., Havenstrite, K.L., Magnusson, K.E., Sacco, A., Leonardi, N.A., Kraft, P., Nguyen, N.K., Thrun, S., Lutolf, M.P., and Blau, H.M. (2010). Substrate elasticity regulates skeletal muscle stem cell self-renewal in culture. Science *329*, 1078-1081.

Goel, A.J., Rieder, M.K., Arnold, H.H., Radice, G.L., and Krauss, R.S. (2017). Niche Cadherins Control the Quiescence-to-Activation Transition in Muscle Stem Cells. Cell Rep *21*, 2236-2250.

Goetsch, S.C., Hawke, T.J., Gallardo, T.D., Richardson, J.A., and Garry, D.J. (2003). Transcriptional profiling and regulation of the extracellular matrix during muscle regeneration. Physiol Genomics *14*, 261-271.

Gollnick, P.D., and Matoba, H. (1984). The muscle fiber composition of skeletal muscle as a predictor of athletic success. An overview. Am J Sports Med *12*, 212-217.

Gonzales, P.A., Pisitkun, T., Hoffert, J.D., Tchapyjnikov, D., Star, R.A., Kleta, R., Wang, N.S., and Knepper, M.A. (2009). Large-scale proteomics and phosphoproteomics of urinary exosomes. J Am Soc Nephrol *20*, 363-379.

Gould, S.J., Booth, A.M., and Hildreth, J.E. (2003). The Trojan exosome hypothesis. Proc Natl Acad Sci U S A *100*, 10592-10597.

Gould, S.J., and Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles *2*.

Gratchev, A., Guillot, P., Hakiy, N., Politz, O., Orfanos, C.E., Schledzewski, K., and Goerdt, S. (2001). Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. Scand J Immunol *53*, 386-392.

Guescini, M., Canonico, B., Lucertini, F., Maggio, S., Annibalini, G., Barbieri, E., Luchetti, F., Papa, S., and Stocchi, V. (2015). Muscle Releases Alpha-Sarcoglycan Positive Extracellular Vesicles Carrying miRNAs in the Bloodstream. PLoS One *10*, e0125094.

Guescini, M., Guidolin, D., Vallorani, L., Casadei, L., Gioacchini, A.M., Tibollo, P., Battistelli, M., Falcieri, E., Battistin, L., Agnati, L.F*., et al.* (2010). C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction. Exp Cell Res *316*, 1977-1984.

Gunther, S., Kim, J., Kostin, S., Lepper, C., Fan, C.M., and Braun, T. (2013). Myf5-positive satellite cells contribute to Pax7-dependent long-term maintenance of adult muscle stem cells. Cell Stem Cell *13*, 590-601.

Hägerstrand H, Mrówczyńska L, Salzer U, Prohaska R, Michelsen KA, Kralj-Iglic V, Iglic A. (2006). Curvature-dependent lateral distribution of raft markers in the human erythrocyte membrane. Mol Membr Biol. 2006 May-Jun;23(3):277-88.

Hashimoto, N., Murase, T., Kondo, S., Okuda, A., and Inagawa-Ogashiwa, M. (2004). Muscle reconstitution by muscle satellite cell descendants with stem cell-like properties. Development *131*, 5481-5490.

Haugen, F., Norheim, F., Lian, H., Wensaas, A.J., Dueland, S., Berg, O., Funderud, A., Skalhegg, B.S., Raastad, T., and Drevon, C.A. (2010). IL-7 is expressed and secreted by human skeletal muscle cells. Am J Physiol Cell Physiol *298*, C807-816.

Hemler, M.E. (2005). Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol *6*, 801-811.

Hemler, M.E. (2008). Targeting of tetraspanin proteins--potential benefits and strategies. Nat Rev Drug Discov *7*, 747-758.

Heredia, J.E., Mukundan, L., Chen, F.M., Mueller, A.A., Deo, R.C., Locksley, R.M., Rando, T.A., and Chawla, A. (2013). Type 2 innate signals stimulate fibro/adipogenic progenitors to facilitate muscle regeneration. Cell *153*, 376-388.

Hernandez, J.L., Davda, D., Majmudar, J.D., Won, S.J., Prakash, A., Choi, A.I., and Martin, B.R. (2016). Correlated S-palmitoylation profiling of Snail-induced epithelial to mesenchymal transition. Mol Biosyst *12*, 1799-1808.

Hirai, M., Iwase, H., Hayakawa, T., Koizumi, M., and Takahashi, H. (2003). Determination of asymmetric structure of ganglioside-DPPC mixed vesicle using SANS, SAXS, and DLS. Biophys J *85*, 1600-1610.

Hunt, L.C., Upadhyay, A., Jazayeri, J.A., Tudor, E.M., and White, J.D. (2013). An anti-inflammatory role for leukemia inhibitory factor receptor signaling in regenerating skeletal muscle. Histochem Cell Biol *139*, 13-34.

Hurley, J.H., and Odorizzi, G. (2012). Get on the exosome bus with ALIX. Nat Cell Biol *14*, 654-655.

Ibrahim, A., Neinast, M., and Arany, Z.P. (2017). Myobolites: muscle-derived metabolites with paracrine and systemic effects. Curr Opin Pharmacol *34*, 15-20.

Irintchev, A., Zeschnigk, M., Starzinski-Powitz, A., and Wernig, A. (1994). Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles. Dev Dyn *199*, 326-337.

Ishido, M., and Kasuga, N. (2015). Characteristics of the Localization of Connexin 43 in Satellite Cells during Skeletal Muscle Regeneration In Vivo. Acta Histochem Cytochem *48*, 53-60.

Jimenez, A.J., Maiuri, P., Lafaurie-Janvore, J., Divoux, S., Piel, M., and Perez, F. (2014). ESCRT machinery is required for plasma membrane repair. Science *343*, 1247136.

Joe, A.W., Yi, L., Natarajan, A., Le Grand, F., So, L., Wang, J., Rudnicki, M.A., and Rossi, F.M. (2010). Muscle injury activates resident fibro/adipogenic progenitors that facilitate myogenesis. Nat Cell Biol *12*, 153-163.

Johnsen, K.B., Gudbergsson, J.M., Skov, M.N., Pilgaard, L., Moos, T., and Duroux, M. (2014). A comprehensive overview of exosomes as drug delivery vehicles - endogenous nanocarriers for targeted cancer therapy. Biochim Biophys Acta *1846*, 75-87.

Juliano, R.L. (2002). Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. Annu Rev Pharmacol Toxicol *42*, 283-323.

Kalra, H., Drummen, G.P., and Mathivanan, S. (2016). Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. Int J Mol Sci *17*, 170.

Keerthikumar, S., Chisanga, D., Ariyaratne, D., Al Saffar, H., Anand, S., Zhao, K., Samuel, M., Pathan, M., Jois, M., Chilamkurti, N.*, et al.* (2016). ExoCarta: A Web-Based Compendium of Exosomal Cargo. J Mol Biol *428*, 688-692.

Kim, H.J., Higashimori, T., Park, S.Y., Choi, H., Dong, J., Kim, Y.J., Noh, H.L., Cho, Y.R., Cline, G., Kim, Y.B.*, et al.* (2004). Differential effects of interleukin-6 and -10 on skeletal muscle and liver insulin action in vivo. Diabetes *53*, 1060-1067.

Kim, J., Sitaraman, S., Hierro, A., Beach, B.M., Odorizzi, G., and Hurley, J.H. (2005). Structural basis for endosomal targeting by the Bro1 domain. Dev Cell *8*, 937-947.

Kim, S.M., and Kim, H.S. (2017). Engineering of extracellular vesicles as drug delivery vehicles. Stem Cell Investig *4*, 74.

Koch, U., Lehal, R., and Radtke, F. (2013). Stem cells living with a Notch. Development *140*, 689-704.

Kooijmans, S.A.A., Schiffelers, R.M., Zarovni, N., and Vago, R. (2016). Modulation of tissue tropism and biological activity of exosomes and other extracellular vesicles: New nanotools for cancer treatment. Pharmacol Res *111*, 487-500.

Koopman, R., Ly, C.H., and Ryall, J.G. (2014). A metabolic link to skeletal muscle wasting and regeneration. Front Physiol *5*, 32.

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J.P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., and Thery, C. (2016). Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. Proc Natl Acad Sci U S A *113*, E968-977.

Kowal, J., Tkach, M., and Thery, C. (2014). Biogenesis and secretion of exosomes. Curr Opin Cell Biol *29*, 116-125.

Krause, C., Guzman, A., and Knaus, P. (2011). Noggin. Int J Biochem Cell Biol *43*, 478-481.

Kuang, S., Kuroda, K., Le Grand, F., and Rudnicki, M.A. (2007). Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell *129*, 999-1010.

Kunadt, M., Eckermann, K., Stuendl, A., Gong, J., Russo, B., Strauss, K., Rai, S., Kugler, S., Falomir Lockhart, L., Schwalbe, M.*, et al.* (2015). Extracellular vesicle sorting of alpha-Synuclein is regulated by sumoylation. Acta Neuropathol *129*, 695-713.

Kurek, J.B., Bower, J.J., Romanella, M., Koentgen, F., Murphy, M., and Austin, L. (1997). The role of leukemia inhibitory factor in skeletal muscle regeneration. Muscle Nerve *20*, 815-822.

Lai, R.C., Yeo, R.W., Tan, K.H., and Lim, S.K. (2013). Exosomes for drug delivery - a novel application for the mesenchymal stem cell. Biotechnol Adv *31*, 543-551.

Le Bihan, M.C., Bigot, A., Jensen, S.S., Dennis, J.L., Rogowska-Wrzesinska, A., Laine, J., Gache, V., Furling, D., Jensen, O.N., Voit, T.*, et al.* (2012). In-depth analysis of the secretome identifies three major independent secretory pathways in differentiating human myoblasts. J Proteomics *77*, 344-356.

Levental, I., Lingwood, D., Grzybek, M., Coskun, U., and Simons, K. (2010). Palmitoylation regulates raft affinity for the majority of integral raft proteins. Proc Natl Acad Sci U S A *107*, 22050-22054.

Li, S., Li, J., Ning, L., Wang, S., Niu, Y., Jin, N., Yao, X., Liu, H., and Xi, L. (2015). In Silico Identification of Protein S-Palmitoylation Sites and Their Involvement in Human Inherited Disease. J Chem Inf Model *55*, 2015-2025.

Liu, H., Niu, A., Chen, S.E., and Li, Y.P. (2011). Beta3-integrin mediates satellite cell differentiation in regenerating mouse muscle. FASEB J *25*, 1914-1921.

Liu, W., Wen, Y., Bi, P., Lai, X., Liu, X.S., Liu, X., and Kuang, S. (2012). Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. Development *139*, 2857-2865.

Luque, E., Pena, J., Martin, P., Jimena, I., and Vaamonde, R. (1995). Capillary supply during development of individual regenerating muscle fibers. Anat Histol Embryol *24*, 87-89.

Machado, L., Esteves de Lima, J., Fabre, O., Proux, C., Legendre, R., Szegedi, A., Varet, H., Ingerslev, L.R., Barres, R., Relaix, F.*, et al.* (2017). In Situ Fixation Redefines Quiescence and Early Activation of Skeletal Muscle Stem Cells. Cell Rep *21*, 1982-1993.

Mahul-Mellier, A.L., Hemming, F.J., Blot, B., Fraboulet, S., and Sadoul, R. (2006). Alix, making a link between apoptosis-linked gene-2, the endosomal sorting complexes required for transport, and neuronal death in vivo. J Neurosci *26*, 542-549.

Marthiens, V., Kazanis, I., Moss, L., Long, K., and Ffrench-Constant, C. (2010). Adhesion molecules in the stem cell niche--more than just staying in shape? J Cell Sci *123*, 1613-1622.

Martin-Serrano, J., Yarovoy, A., Perez-Caballero, D., and Bieniasz, P.D. (2003). Divergent retroviral late-budding domains recruit vacuolar protein sorting factors by using alternative adaptor proteins. Proc Natl Acad Sci U S A *100*, 12414-12419.

Mathew, S.J., Hansen, J.M., Merrell, A.J., Murphy, M.M., Lawson, J.A., Hutcheson, D.A., Hansen, M.S., Angus-Hill, M., and Kardon, G. (2011). Connective tissue fibroblasts and Tcf4 regulate myogenesis. Development *138*, 371-384.

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Faure, J., Blanc, N.S., Matile, S., Dubochet, J., Sadoul, R.*, et al.* (2004). Role of LBPA and Alix in multivesicular liposome formation and endosome organization. Science *303*, 531-534.

Matsuzaka, Y., Tanihata, J., Komaki, H., Ishiyama, A., Oya, Y., Ruegg, U., Takeda, S.I., and Hashido, K. (2016). Characterization and Functional Analysis of Extracellular Vesicles and Muscle-Abundant miRNAs (miR-1, miR-133a, and miR-206) in C2C12 Myocytes and mdx Mice. PLoS One *11*, e0167811.

Mauro, A. (1961). Satellite cell of skeletal muscle fibers. J Biophys Biochem Cytol *9*, 493-495.

Mercier, V., Laporte, M.H., Destaing, O., Blot, B., Blouin, C.M., Pernet-Gallay, K., Chatellard, C., Saoudi, Y., Albiges-Rizo, C., Lamaze, C.*, et al.* (2016). ALG-2 interacting protein-X (Alix) is essential for clathrin-independent endocytosis and signaling. Sci Rep *6*, 26986.

Miller, I.V., and Grunewald, T.G. (2015). Tumour-derived exosomes: Tiny envelopes for big stories. Biol Cell *107*, 287-305.

Mitchell, K.J., Pannerec, A., Cadot, B., Parlakian, A., Besson, V., Gomes, E.R., Marazzi, G., and Sassoon, D.A. (2010). Identification and characterization of a non-satellite cell muscle resident progenitor during postnatal development. Nat Cell Biol *12*, 257-266.

Moreno-Gonzalo, O., Fernandez-Delgado, I., and Sanchez-Madrid, F. (2017). Post-translational add-ons mark the path in exosomal protein sorting. Cell Mol Life Sci.

Morita, E., Colf, L.A., Karren, M.A., Sandrin, V., Rodesch, C.K., and Sundquist, W.I. (2010). Human ESCRT-III and VPS4 proteins are required for centrosome and spindle maintenance. Proc Natl Acad Sci U S A *107*, 12889-12894.

Mourikis, P., Sambasivan, R., Castel, D., Rocheteau, P., Bizzarro, V., and Tajbakhsh, S. (2012). A critical requirement for notch signaling in maintenance of the quiescent skeletal muscle stem cell state. Stem Cells *30*, 243-252.

Mozzetta, C., Consalvi, S., Saccone, V., Tierney, M., Diamantini, A., Mitchell, K.J., Marazzi, G., Borsellino, G., Battistini, L., Sassoon, D*., et al.* (2013). Fibroadipogenic progenitors mediate the ability of HDAC inhibitors to promote regeneration in dystrophic muscles of young, but not old Mdx mice. EMBO Mol Med *5*, 626-639.

Munshi, U.M., Kim, J., Nagashima, K., Hurley, J.H., and Freed, E.O. (2007). An Alix fragment potently inhibits HIV-1 budding: characterization of binding to retroviral YPXL late domains. J Biol Chem *282*, 3847-3855.

Murphy, M.M., Lawson, J.A., Mathew, S.J., Hutcheson, D.A., and Kardon, G. (2011). Satellite cells, connective tissue fibroblasts and their interactions are crucial for muscle regeneration. Development *138*, 3625-3637.

Nabhan, J.F., Hu, R., Oh, R.S., Cohen, S.N., and Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. Proc Natl Acad Sci U S A *109*, 4146-4151.

Nakamura, Y., Miyaki, S., Ishitobi, H., Matsuyama, S., Nakasa, T., Kamei, N., Akimoto, T., Higashi, Y., and Ochi, M. (2015). Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. FEBS Lett *589*, 1257-1265.

Natarajan, A., Lemos, D.R., and Rossi, F.M. (2010). Fibro/adipogenic progenitors: a double-edged sword in skeletal muscle regeneration. Cell Cycle *9*, 2045-2046.

Nickels, J.D., Chatterjee, S., Stanley, C.B., Qian, S., Cheng, X., Myles, D.A.A., Standaert, R.F., Elkins, J.G., and Katsaras, J. (2017). The in vivo structure of biological membranes and evidence for lipid domains. PLoS Biol *15*, e2002214.

Odorizzi, G. (2006). The multiple personalities of Alix. J Cell Sci *119*, 3025-3032.

Oeste, C.L., Pinar, M., Schink, K.O., Martinez-Turrion, J., Stenmark, H., Penalva, M.A., and Perez-Sala, D. (2014). An isoprenylation and palmitoylation motif promotes intraluminal vesicle delivery of proteins in cells from distant species. PLoS One *9*, e107190.

Ollitrault D, B.V., Correra R, Hoareau B, Pöhle-Jronawitter S, Stricker S, Marazzi G, Sassoon D (2017). Glucose-deprivation induces interstitial progenitor vasculogenesis (Submitted).

Ono, Y., Calhabeu, F., Morgan, J.E., Katagiri, T., Amthor, H., and Zammit, P.S. (2011). BMP signalling permits population expansion by preventing premature myogenic differentiation in muscle satellite cells. Cell Death Differ *18*, 222-234.

Osteikoetxea, X., Balogh, A., Szabo-Taylor, K., Nemeth, A., Szabo, T.G., Paloczi, K., Sodar, B., Kittel, A., Gyorgy, B., Pallinger, E*., et al.* (2015). Improved characterization of EV preparations based on protein to lipid ratio and lipid properties. PLoS One *10*, e0121184.

Pan, S., Wang, R., Zhou, X., He, G., Koomen, J., Kobayashi, R., Sun, L., Corvera, J., Gallick, G.E., and Kuang, J. (2006). Involvement of the conserved adaptor protein Alix in actin cytoskeleton assembly. J Biol Chem *281*, 34640-34650.

Pannerec, A., Marazzi, G., and Sassoon, D. (2012). Stem cells in the hood: the skeletal muscle niche. Trends Mol Med *18*, 599-606.

Pawlikowski, B., Vogler, T.O., Gadek, K., and Olwin, B.B. (2017). Regulation of skeletal muscle stem cells by fibroblast growth factors. Dev Dyn *246*, 359-367.

Pedersen, B.K. (2013). Muscle as a secretory organ. Compr Physiol *3*, 1337-1362.

Pedersen, B.K., and Febbraio, M.A. (2008). Muscle as an endocrine organ: focus on muscle-derived interleukin-6. Physiol Rev *88*, 1379-1406.

Peinado, H., Aleckovic, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajar, C.*, et al.* (2012). Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. Nat Med *18*, 883-891.

Perez-Hernandez, D., Gutierrez-Vazquez, C., Jorge, I., Lopez-Martin, S., Ursa, A., Sanchez-Madrid, F., Vazquez, J., and Yanez-Mo, M. (2013). The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes. J Biol Chem *288*, 11649-11661.

Pires, R., Hartlieb, B., Signor, L., Schoehn, G., Lata, S., Roessle, M., Moriscot, C., Popov, S., Hinz, A., Jamin, M.*, et al.* (2009). A crescent-shaped ALIX dimer targets ESCRT-III CHMP4 filaments. Structure *17*, 843-856.

Podesta, A., Tiana, G., Milani, P., and Manno, M. (2006). Early events in insulin fibrillization studied by time-lapse atomic force microscopy. Biophys J *90*, 589-597.

Rana S., Z.M. (2013). The Functional Importance of Tetraspanins in Exosomes. In Emerging Concepts of Tumor Exosome–Mediated Cell-Cell Communication (Springer, New York, NY).

Rao, R.V., Poksay, K.S., Castro-Obregon, S., Schilling, B., Row, R.H., del Rio, G., Gibson, B.W., Ellerby, H.M., and Bredesen, D.E. (2004). Molecular components of a cell death pathway activated by endoplasmic reticulum stress. J Biol Chem *279*, 177-187.

Raposo, G., Nijman, H.W., Stoorvogel, W., Liejendekker, R., Harding, C.V., Melief, C.J., and Geuze, H.J. (1996). B lymphocytes secrete antigen-presenting vesicles. J Exp Med *183*, 1161-1172.

Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., Reca, R., Dvorak, P., and Ratajczak, M.Z. (2006). Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia *20*, 847-856.

Relaix, F., and Marcelle, C. (2009). Muscle stem cells. Curr Opin Cell Biol *21*, 748-753.

Ren, W., Jhala, U.S., and Du, K. (2013). Proteomic analysis of protein palmitoylation in adipocytes. Adipocyte *2*, 17-28.

Rojas-Rios, P., and Gonzalez-Reyes, A. (2014). Concise review: The plasticity of stem cell niches: a general property behind tissue homeostasis and repair. Stem Cells *32*, 852-859.

Romancino, D.P., Paterniti, G., Campos, Y., De Luca, A., Di Felice, V., d'Azzo, A., and Bongiovanni, A. (2013). Identification and characterization of the nano-sized vesicles released by muscle cells. FEBS Lett *587*, 1379-1384.

Ross-Murphy, S.B. (1977). Dynamic Light Scattering. B. J. Berne and R. Pecora, John Wiley, New York, 1976,. Brit Poly J.

Rustom, A., Saffrich, R., Markovic, I., Walther, P., and Gerdes, H.H. (2004). Nanotubular highways for intercellular organelle transport. Science *303*, 1007-1010.

Salaun, C., Greaves, J., and Chamberlain, L.H. (2010). The intracellular dynamic of protein palmitoylation. J Cell Biol *191*, 1229-1238.

Sanders, S.S., Martin, D.D., Butland, S.L., Lavallee-Adam, M., Calzolari, D., Kay, C., Yates, J.R., 3rd, and Hayden, M.R. (2015). Curation of the Mammalian Palmitoylome Indicates a Pivotal Role for Palmitoylation in Diseases and Disorders of the Nervous System and Cancers. PLoS Comput Biol *11*, e1004405.

Sanderson, R.D., Bandari, S.K., and Vlodavsky, I. (2017). Proteases and glycosidases on the surface of exosomes: Newly discovered mechanisms for extracellular remodeling. Matrix Biol.

Sandonà M, C.S., Tucciarone L, De Bardi M, Scimeca M, Angelini D, Buffa V, D'Amico A, Bertini E, Bouché M, Bongiovanni A, Puri PL and Saccone V (2017). HDAC inhibitors Modulate microRNA Content of Fibroadipogenic Progenitor-derived Exosomes to Promote Regeneration and Inhibit Fibrosis of Dystrophic Muscles. (Cell Stem Cell, under revision).

Sassoon, D., Lyons, G., Wright, W.E., Lin, V., Lassar, A., Weintraub, H., and Buckingham, M. (1989). Expression of two myogenic regulatory factors myogenin and MyoD1 during mouse embryogenesis. Nature *341*, 303-307.

Scheffer, L.L., Sreetama, S.C., Sharma, N., Medikayala, S., Brown, K.J., Defour, A., and Jaiswal, J.K. (2014). Mechanism of Ca(2)(+)-triggered ESCRT assembly and regulation of cell membrane repair. Nat Commun *5*, 5646.

Schmidt, M.H., Dikic, I., and Bogler, O. (2005). Src phosphorylation of Alix/AIP1 modulates its interaction with binding partners and antagonizes its activities. J Biol Chem *280*, 3414-3425.

Schmidt, M.H., Hoeller, D., Yu, J., Furnari, F.B., Cavenee, W.K., Dikic, I., and Bogler, O. (2004). Alix/AIP1 antagonizes epidermal growth factor receptor downregulation by the Cbl-SETA/CIN85 complex. Mol Cell Biol *24*, 8981-8993.

Serrano, A.L., Baeza-Raja, B., Perdiguero, E., Jardi, M., and Munoz-Canoves, P. (2008). Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab *7*, 33-44.

Serwa, R.A., Abaitua, F., Krause, E., Tate, E.W., and O'Hare, P. (2015). Systems Analysis of Protein Fatty Acylation in Herpes Simplex Virus-Infected Cells Using Chemical Proteomics. Chem Biol *22*, 1008-1017.

Shea, K.L., Xiang, W., LaPorta, V.S., Licht, J.D., Keller, C., Basson, M.A., and Brack, A.S. (2010). Sprouty1 regulates reversible quiescence of a self-renewing adult muscle stem cell pool during regeneration. Cell Stem Cell *6*, 117-129.

Shen, B., Wu, N., Yang, J.M., and Gould, S.J. (2011). Protein targeting to exosomes/microvesicles by plasma membrane anchors. J Biol Chem *286*, 14383-14395.

Sidhu, S.S., Mengistab, A.T., Tauscher, A.N., LaVail, J., and Basbaum, C. (2004). The microvesicle as a vehicle for EMMPRIN in tumor-stromal interactions. Oncogene *23*, 956-963.

Simons, M., and Raposo, G. (2009). Exosomes--vesicular carriers for intercellular communication. Curr Opin Cell Biol *21*, 575-581.

Skog, J., Wurdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T., Jr., Carter, B.S., Krichevsky, A.M., and Breakefield, X.O. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol *10*, 1470-1476.

Steensberg, A., Fischer, C.P., Keller, C., Moller, K., and Pedersen, B.K. (2003). IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans. Am J Physiol Endocrinol Metab *285*, E433-437.

Steensberg, A., van Hall, G., Osada, T., Sacchetti, M., Saltin, B., and Klarlund Pedersen, B. (2000). Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. J Physiol *529 Pt 1*, 237-242.

Stein, J.M., and Luzio, J.P. (1991). Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. Biochem J *274 (Pt 2)*, 381-386.

Strack, B., Calistri, A., Craig, S., Popova, E., and Gottlinger, H.G. (2003). AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. Cell *114*, 689-699.

Stuffers, S., Sem Wegner, C., Stenmark, H., and Brech, A. (2009). Multivesicular endosome biogenesis in the absence of ESCRTs. Traffic *10*, 925-937.

Stukelj, R., Schara, K., Bedina-Zavec, A., Sustar, V., Pajnic, M., Paden, L., Krek, J.L., Kralj-Iglic, V., Mrvar-Brecko, A., and Jansa, R. (2017). Effect of shear stress in the flow through the sampling needle on concentration of nanovesicles isolated from blood. Eur J Pharm Sci *98*, 17-29.

Sun, S., Sun, L., Zhou, X., Wu, C., Wang, R., Lin, S.H., and Kuang, J. (2016). Phosphorylation-Dependent Activation of the ESCRT Function of ALIX in Cytokinetic Abscission and Retroviral Budding. Dev Cell *37*, 581.

Sustar, V., Bedina-Zavec, A., Stukelj, R., Frank, M., Bobojevic, G., Jansa, R., Ogorevc, E., Kruljc, P., Mam, K., Simunic, B.*, et al.* (2011). Nanoparticles isolated from blood: a reflection of vesiculability of blood cells during the isolation process. Int J Nanomedicine *6*, 2737-2748.

Takada, S., Okita, K., Suga, T., Omokawa, M., Kadoguchi, T., Sato, T., Takahashi, M., Yokota, T., Hirabayashi, K., Morita, N.*, et al.* (2012). Low-intensity exercise can increase muscle mass and strength proportionally to enhanced metabolic stress under ischemic conditions. J Appl Physiol (1985) *113*, 199-205.

Tan, S.S., Yin, Y., Lee, T., Lai, R.C., Yeo, R.W., Zhang, B., Choo, A., and Lim, S.K. (2013). Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane. J Extracell Vesicles *2*.

Tatsumi, R., Hattori, A., Ikeuchi, Y., Anderson, J.E., and Allen, R.E. (2002). Release of hepatocyte growth factor from mechanically stretched skeletal muscle satellite cells and role of pH and nitric oxide. Mol Biol Cell *13*, 2909-2918.

Tavi, P., Korhonen, T., Hanninen, S.L., Bruton, J.D., Loof, S., Simon, A., and Westerblad, H. (2010). Myogenic skeletal muscle satellite cells communicate by tunnelling nanotubes. J Cell Physiol *223*, 376-383.

Thery, C., Amigorena, S., Raposo, G., and Clayton, A. (2006). Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol *Chapter 3*, Unit 3 22.

Thery, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. Nat Rev Immunol *2*, 569-579.

Thuma, F., Heiler, S., Schnolzer, M., and Zoller, M. (2016). Palmitoylated claudin7 captured in glycolipid-enriched membrane microdomains promotes metastasis via associated transmembrane and cytosolic molecules. Oncotarget *7*, 30659-30677.

Tomasek, J.J., Gabbiani, G., Hinz, B., Chaponnier, C., and Brown, R.A. (2002). Myofibroblasts and mechano-regulation of connective tissue remodelling. Nat Rev Mol Cell Biol *3*, 349-363.

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B., and Simons, M. (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science *319*, 1244-1247.

Trapnell, C., Pachter, L., and Salzberg, S.L. (2009). TopHat: discovering splice junctions with RNA-Seq. Bioinformatics *25*, 1105-1111.

Uezumi, A., Fukada, S., Yamamoto, N., Takeda, S., and Tsuchida, K. (2010). Mesenchymal progenitors distinct from satellite cells contribute to ectopic fat cell formation in skeletal muscle. Nat Cell Biol *12*, 143-152.

Van Engelenburg, S.B., Shtengel, G., Sengupta, P., Waki, K., Jarnik, M., Ablan, S.D., Freed, E.O., Hess, H.F., and Lippincott-Schwartz, J. (2014). Distribution of ESCRT machinery at HIV assembly sites reveals virus scaffolding of ESCRT subunits. Science *343*, 653-656.

van Niel, G., Raposo, G., Candalh, C., Boussac, M., Hershberg, R., Cerf-Bensussan, N., and Heyman, M. (2001). Intestinal epithelial cells secrete exosome-like vesicles. Gastroenterology *121*, 337-349.

von Maltzahn, J., Jones, A.E., Parks, R.J., and Rudnicki, M.A. (2013). Pax7 is critical for the normal function of satellite cells in adult skeletal muscle. Proc Natl Acad Sci U S A *110*, 16474-16479.

Wang, H., Noulet, F., Edom-Vovard, F., Tozer, S., Le Grand, F., and Duprez, D. (2010). Bmp signaling at the tips of skeletal muscles regulates the number of fetal muscle progenitors and satellite cells during development. Dev Cell *18*, 643-654.

Wiklander, O.P., Nordin, J.Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mager, I., Vader, P., Lee, Y., Sork, H., Seow, Y.*, et al.* (2015). Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. J Extracell Vesicles *4*, 26316.

Wilschut, K.J., van Tol, H.T., Arkesteijn, G.J., Haagsman, H.P., and Roelen, B.A. (2011). Alpha 6 integrin is important for myogenic stem cell differentiation. Stem Cell Res *7*, 112-123.

Wollert, T., and Hurley, J.H. (2010). Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. Nature *464*, 864-869.

Wrigley, J.D., Ahmed, T., Nevett, C.L., and Findlay, J.B. (2000). Peripherin/rds influences membrane vesicle morphology. Implications for retinopathies. J Biol Chem *275*, 13191-13194.

Xian, X., Gopal, S., and Couchman, J.R. (2010). Syndecans as receptors and organizers of the extracellular matrix. Cell Tissue Res *339*, 31-46.

Yanez-Mo, M., Barreiro, O., Gordon-Alonso, M., Sala-Valdes, M., and Sanchez-Madrid, F. (2009). Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. Trends Cell Biol *19*, 434-446.

Yanez-Mo, M., Siljander, P.R., Andreu, Z., Zavec, A.B., Borras, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J.*, et al.* (2015). Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles *4*, 27066.

Yang, J.M., and Gould, S.J. (2013). The cis-acting signals that target proteins to exosomes and microvesicles. Biochem Soc Trans *41*, 277-282.

Yang, W., Di Vizio, D., Kirchner, M., Steen, H., and Freeman, M.R. (2010). Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes. Mol Cell Proteomics *9*, 54-70.

Yin, H., Price, F., and Rudnicki, M.A. (2013). Satellite cells and the muscle stem cell niche. Physiol Rev *93*, 23-67.

Yoshida, N., Yoshida, S., Koishi, K., Masuda, K., and Nabeshima, Y. (1998). Cell heterogeneity upon myogenic differentiation: down-regulation of MyoD and Myf-5 generates 'reserve cells'. J Cell Sci *111 (Pt 6)*, 769-779.

Zammit, P.S., Golding, J.P., Nagata, Y., Hudon, V., Partridge, T.A., and Beauchamp, J.R. (2004). Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? J Cell Biol *166*, 347-357.

Zammit, P.S., Partridge, T.A., and Yablonka-Reuveni, Z. (2006). The skeletal muscle satellite cell: the stem cell that came in from the cold. J Histochem Cytochem *54*, 1177-1191.

Zhai, Q., Landesman, M.B., Chung, H.Y., Dierkers, A., Jeffries, C.M., Trewhella, J., Hill, C.P., and Sundquist, W.I. (2011a). Activation of the retroviral budding factor ALIX. J Virol *85*, 9222-9226.

Zhai, Q., Landesman, M.B., Robinson, H., Sundquist, W.I., and Hill, C.P. (2011b). Structure of the Bro1 domain protein BROX and functional analyses of the ALIX Bro1 domain in HIV-1 budding. PLoS One *6*, e27466.

Zhang, C., Li, Y., Wu, Y., Wang, L., Wang, X., and Du, J. (2013). Interleukin-6/signal transducer and activator of transcription 3 (STAT3) pathway is essential for macrophage infiltration and myoblast proliferation during muscle regeneration. J Biol Chem *288*, 1489-1499.

APPENDIX I

CURRICULUM VITAE

Curriculum Vitae

Personal Information

Valentina Buffa

Date of birth	23/Jan/1988		
Nationality	Italian		
Address	Via Baronia 2 - 91010 Vita (Trapani) ITALY		
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Institutional Information

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Advisors Information

Antonella Bongiovanni, Ph.D. (IBIM, C.N.R.) Palermo, ITALY bongiovanni@ibim.cnr.it

David Sassoon, PhD and Giovanna Marazzi, MD (ICAN, U1166, UPMC) Paris, FRANCE
david.a.sassoon@gmail.com | giovanna.em.marazzi@gmail.com

Education

Since 01/2015	PhD student of the doctoral program Molecular Medicine and Biotechnologies at Dipartimento di Biopatologia e Biotecnologie Mediche, Università degli studi di Palermo, ITALY PhD project: “Intercellular Communication in Skeletal Muscle Stem Cell Niche: Focus on extracellular vesicles and secreted signals”, supervision Antonella Bongiovanni, Ph.D. (IBIM, C.N.R.) Palermo, ITALY and co-supervision David Sassoon, PhD and Giovanna Marazzi, MD (ICAN, U1166, UPMC) Paris, FRANCE. (degree expected for 03/2018).
09/2011 - 10/2014	Master degree in Medical Biotechnologies and Molecular Medicine at Facoltà di Medicina, Università degli studi di Palermo, ITALY Thesis “Culture conditions for successful ex vivo lentiviral transduction of hematopoietic stem cells” supervision Professor Gerard Wagemaker at Stem cell gene therapy lab, Hematology department, Erasmus Medical Center, Rotterdam, THE NERTHERLANDS. Final grade 110/110 cum laude.
09/2007 - 07/2011	Undergraduate degree in Biotechnologies with expertise in biomedicine at Facoltà di Scienze MM.FF.NN., Università degli studi di Palermo, ITALY Thesis “Ex vivo hematopoietic stem cells expansion and trans-infection efficiency with lentiviral vectors”, supervision Santina Acuto, PhD at Terapia Genica lab, Ematologia

II, Ospedali Riuniti Villa Sofia–Cervello, Palermo, ITALY. Final grade 108/110.

Professional Experience

12/2014 - 4/2015 — Short-term contract: “Mutations screening for cystic fibrosis and Congenital dyserythropoietic anemia (CDA), supervision of Dott.ssa Elena D'Alcamo at Campus of Hematology Franco e Piera Cutino - A.O.O.R. Villa Sofia-Cervello, Palermo, ITALY.

02/2014 - 07/2014 — Fellow in the research project “Characterization of nano and microvesiscles released by skeletal muscle cells; nanotechnology-based diagnostics in neurological diseases and experimental oncology – NADINE | Nanomax”, supervision Antonella Bongiovanni, Ph.D. (IBIM, C.N.R.) Palermo, ITALY.

07/2012 - 12/2012 — Internship in the context of RNA binding protein study in neuronal cell differentiation, supervision Professor Italia Di Liegro e Gabriella Schiera, PhD at Dipartimento STEBICEF, Università degli studi di Palermo, ITALY.

04/2012 - 06/2012 — Internship in the context of the study of sea urchin as in vivo model for the screening of bioactive molecules with effects on epithelial mesenchymal transition, supervision Antonella Bongiovanni, Ph.D. (IBIM, C.N.R.) Palermo, ITALY.

Publications and Manuscripts

Manuscript — *Identification of a tissue-resident progenitor population with vascular cell fate potential.* Ollitrault D, **Buffa V**, Correra R, Hoareau B, Pöhle-Jronawitter S, Stricker S, Marazzi M and Sassoon D.

Manuscript — *Palmitoylation is a post-translational modification of Alix regulating the membrane organization of exosome*. Romancino DP*, **Buffa V***, Ferrara I, Raccosta S, Notaro A, Caruso S, Noto R, Martonara V, Cupane A, Giallongo A, d'Azzo A, Manno M, Bongiovanni A. * equal contribution

Under revision — *HDAC inhibitors Modulate microRNA Content of Fibroadipogenic Progenitor-derived Exosomes to Promote Regeneration and Inhibit Fibrosis of Dystrophic Muscles*. Sandonà M, Consalvi S, Tucciarone L, De Bardi M, Scimeca M, Angelini D, **Buffa V**, D’Amico A, Bertini E, Bouché M, Bongiovanni A, Puri PL* and Saccone V* *Cell Stem Cell, 2017*

2017 — *A sea urchin in vivo model to evaluate Epithelial-Mesenchymal Transition*. Romancino DP, Anello L, Lavanco A, **Buffa V**, Di Bernardo M, Bongiovanni A. *Dev Growth Differ, 2017*

2016 — *New Codanin-1 Gene Mutations in an Italian Patient with Congenital Dyserythropoietic Anemia Type I and Heterozygous Beta-Thalassemia.* D’Alcamo E, Agrigento V, Pitrolo L, Sclafani S, Barone R, Calvaruso G, **Buffa V**, Maggio A. *Case Report, Indian J Hematol Blood Transfus, 2016*

Communications in Scientific meetings

Oral communications

26 - 28 May 2016 — **ABCD Meeting - Organelle Biogenesis and Signal Transduction - Turin, ITALY**
“Importance of protein lipidation in the production and structural organization of extracellular vesicles (EVs): an integrated approach in skeletal muscle cells”
Buffa V, Romancino DP, Raccosta S, Notaro A, Ferrara I, d'Azzo A, Martorana V, Manno M, Bongiovanni A.

Poster Presentations

28 September – 02 October 2015 — **FisMat 2015 - Italian National Conference on Condensed Matter Physics Palermo, ITALY**
"Characterization of skeletal muscle-derived extracellular vesicles (EVs): the effect of palmitoylation in EV biogenesis and the structural organization of their membrane"
Buffa V, Raccosta S, Romancino D, Notaro A, Noto R, Martorana V, Manno M, Bongiovanni A.

2 - 4 July 2014 — **I materiali biocompatibili per la medicina - Convegno Nazionale della Società Italiana Biomateriali – Palermo, ITALY**
"Biophysical characterization of skeletal muscle-derived exosomes"
Buffa V, Romancino D, Raccosta S, Lavanco A, Martorana V, Manno M and Bongiovanni A.

26 - 27 June 2014 — **Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo, ITALY**
"Heterogeneity of skeletal muscle-derived extracellular nanovesicles and role of protein lipidation"
Buffa V, Lavanco A, d'Azzo A, Manno M, Romancino D and Bongiovanni A.

Honours and Awards

June 2014 — Best CNR poster of the meeting Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo, ITALY

Fellowships

02/2014 - 07/2014 — Fellowship in the research project NADINE | Nanomax" at Bongiovanni's lab, IBIM, C.N.R., Palermo, ITALY

03/2013 - 09/2013 — Fellowship by Erasmus+ Placement Program to support international internship at Wagemaker's lab, Erasmus Medical Center, Rotterdam, THE NETHERLANDS

Skills and Competences

Languages
Italian (mother tongue)
English (advanced)
French (basic)

Competences
- Human cord blood-derived cells isolation (MACS separation);
- Animal handling:
  - Mice samples collection (bone marrow and muscle);
  - Sea urchin fertilization;
- Cell culture human and mice cells, differentiation and clonogenic assays, transfection and viral vectors production;
- Immunofluorescence assays and confocal microscopy;
- Protein analysis: SDS-page, Western Blot, Acyl Biotin Exchange assay;
- DNA analysis: DNA cloning, PCR and Sanger sequencing, qPCR, reverse dot-blot assay;
- Exosomes purification with differential centrifugations.

References

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APPENDIX II

ORAL COMMUNICATIONS

ABCD Meeting - Organelle Biogenesis and Signal Transduction - Turin, ITALY

"Importance of protein lipidation in the production and structural organization of extracellular vesicles (EVs): an integrated approach in skeletal muscle cells"

Buffa V, Romancino DP, Raccosta S, Notaro A, Ferrara I, d'Azzo A, Martorana V, Manno M, Bongiovanni A.

26 - 28 May 2016

Abstract

Several cell types secrete small vesicles as exosomes containing cell-speci c proteins, lipids

and genetic material. Our aims were to understand how skeletal muscle (SkM) cell generate these vesicles, to evaluate their heterogeneity and their regulation. To characterize exosomes and determine the role of protein lipidation (i.e., S-palmitoylation), we applied an integrated biological/biophysical approach. We were able to determine that Alix (exosomal marker) is S-palmitoylated and that palmitoylation inhibition altered its subcellular localization and protein interaction. We proved that the inhibition of palmitoylation in influences the number, size, heterogeneity of exosomes, as well as the structural organization of their lipid bilayer. us, we propose that S-palmitoylation might regulate the proper function of Alix in SkM EV biogenesis, support the interactions among the exosome regulators and maintain proper EV membrane structural organization. A better understanding of EV biogenesis and function would pave a way for a possible application of SkM-derived exosomes as a novel cell-based therapy for muscle degenerative diseases.

APPENDIX III

POSTER PRESENTATIONS

FisMat 2015 - Italian National Conference on Condensed Matter Physics Palermo, ITALY

"Characterization of skeletal muscle-derived extracellular vesicles (EVs): the effect of palmitoylation in EV biogenesis and the structural organization of their membrane"

Buffa V, Raccosta S, Romancino D, Notaro A, Noto R, Martorana V, Manno M, Bongiovanni A.

28 September – 02 October 2015

I materiali biocompatibili per la medicina - Convegno Nazionale della Società Italiana Biomateriali – Palermo, ITALY

"Biophysical characterization of skeletal muscle-derived exosomes"

Buffa V, Romancino D, Raccosta S, Lavanco A, Martorana V, Manno M and Bongiovanni A.

2 - 4 July 2014

Ricerca di base, interdisciplinare e traslazionale in ambito Biologico e Biotecnologico, Palermo, ITALY

"Heterogeneity of skeletal muscle-derived extracellular nanovesicles and role of protein lipidation"

Buffa V, Lavanco A, d'Azzo A, Manno M, Romancino D and Bongiovanni A.

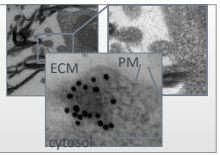
26 - 27 June 2014

Characterization of skeletal muscle-derived extracellular vesicles (EVs): the effect of palmitoylation in EV biogenesis and the structural organization of their membrane.

Valentina Buffa[1], Samuele Raccosta[2], Daniele P. Romancino[1], Antonietta Notaro[1], Rosina Noto[2], Vincenzo Martorana[2], Mauro Manno[2] and Antonella Bongiovanni[1]

[1]Institute of Biomedicine and Molecular Immunology (IBIM), National Research Council (C.N.R.), Palermo, Italy, [2]Institute of Biophysics (IBF), National Research Council (C.N.R.), Palermo, Italy

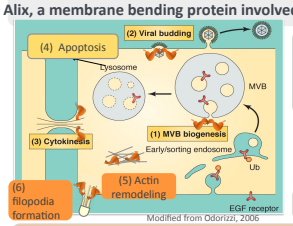
Background: Extracellular vesicles (Evs) are spherical bilayered proteolipids, which are known to harbor a specific subset of bioactive molecules reflecting their originating cell types and conditions. Skeletal muscle (SkM) cells can release Alix-positive Evs (*i.e.*, exosomes), suggesting a new paradigm for understanding how muscles communicate with other organs, such as adipose tissue, bones, the brain, or tumors. Our goal is to understand how muscle cells generate these vesicles and what their regulators are. To this end, we have integrated a biophysical approach (Dynamic Light Scattering, DLS, small-angle X-ray, SAXS, and Nanoparticle Tracking Analysis, NTA) to biological techniques (Acyl Biotin Exchange, ABE) to determine whether protein lipidation (*i.e.*, palmitoylation) affects the localization of EV modulator(s), EV biogenesis, and EV structural organization and heterogeneity.



Question 1. Protein palmitoylation as reversible post-translational modification in the regulation of exosomes biogenesis?

- Different lipidations (i.e. myristoylation, palmitoylation) can target a highly oligomeric recombinant cytoplasmic protein into secreted vesicles (Shen et al., 2011)
- Palmitoylation may specifically contribute to the organization and flexibility of the Tetraspanin Enriched Microdomain (TEM) (Yanez-Mo et al., 2009)
- TEM as sorting platform towards exosomes (Pezer-Hernandez D. et al., 2012)
- Most of the proteins expressed in exosomes present predicted palmitoylation site/s and are prone to oligomerize

Question 2. The multiple personalities of Alix. Alix, a membrane bending protein involved in exosome biogenesis?

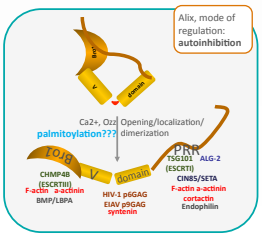


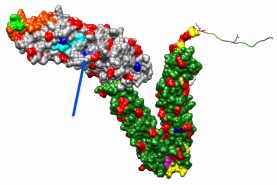
Alix induces a negative mb curvature resulting in 100-nm scale structures:

- (1) MVB biogenesis
- (2) viral budding
- (3) cytokinesis
- (6) filopodia formation

Alix contains a convex face (BRO1) containing a positively charged surface that might function as membrane bending domain to generate a negative curvature

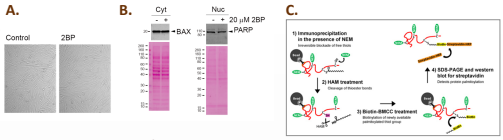
Question 3. Does S-Palmitoylation regulate Alix ?



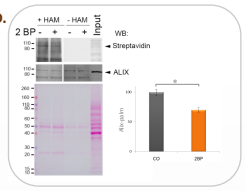


In silico identification of S-palmitoylation sites in huAlix (in BRO1): **Cys 231** (*SeqPalm*)

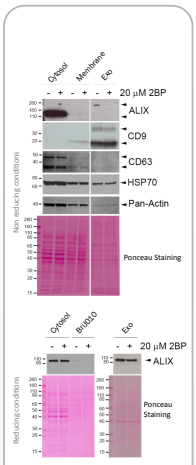
1. Endogenous Alix is palmitoylated in muscle cells



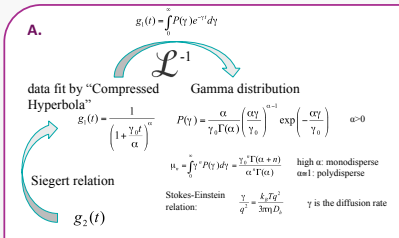
No morphological changes (**A.**), nor apoptotic signs (**B.**) following 2 Bromo Palmitate (2BP) treatment (24 hrs)



IP-ABE assay to detect palmitoylation of Alix protein. Assay scheme **(C.)**. Different levels of palmitoylation after 2BP treatment, immunoprecipitating with anti Alix 3A9 **(D.)**. The open form of Alix is not influenced by 2BP treatment (ABE, using anti-Alix 2H12) **(E.)**.

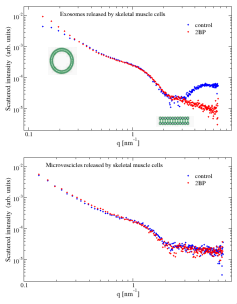


2. 2BP treatments induce a significant increase of the exosome number (DLS: 2x; NTA: 14x!)**(A.)** and of concentration of C2C12-derived exosomal proteins (BCA assays) **(B.)**



3. The 2BP treatments (red) induce an increase in number of vesicles with diameter of 200-400 nm with respect to control (blue)
A. Analysis method of DLS data: from experimental $g_2(t)$ to rate distribution $P(\gamma)$. **B.** Nano-particle Tracking Analysis (dashed) and Dynamic Light Scattering (solid) results on exosomes isolated w/o 2BP treatment.).

3. 2BP treatment induces a loss of the structural order of the exosome membrane in the absence of palmitoylation, but no differences on the microvescicle membrane.
C. Small Angle X-ray Scattering measures on SkM-derived exosomes (up) and microvescicles (down). The scattering intensity curve provides information about the size (low q) and bilayer thickness/ arrangement (high q) of vesicles



4. 2BP treatment induce a significant reduction of Alix and CD9 polimers in exosomes (Exo), as well as a decrease of Alix monomer in the BrijO10-soluble membrane fraction (TEM)

Conclusion: Muscle exosomes could represent a previously undiscovered cellular communication system among muscle cells, which could play an important role in the maintenance of striated muscle homeostasis, regeneration, and repair. The biogenesis of SkM-derived exosomes might be related to Alix and its PTM (lipidation).
Our results show that Alix is palmitoylated in muscle cells and the inhibition of palmitoylation results in: i) increase of the number and diameter of exosomes, ii) modification of the exoxome membrane, and iii) significant decrease of polimeric forms of Alix and CD9 proteins. We propose that palmitoylation might function to regulate the proper function of SkM-derived exosomes.
Future Plans: The role of Alix in the biogenesis of and the heterogeneity of SkM-exosomes will be further confirmed and addressed by characterization of the Alix animal models (generated in d'Azzo lab), and biophysical means (DLS, FCS, and SAXS). Furthermore, we will analyze the function/s of muscle exosomes and palmitoylation in muscle differentiation and repair.

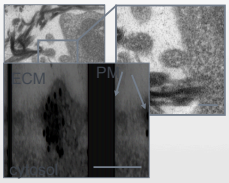
This work was supported by Italian Ministry of Economy and Finance and Ministry of Education, Universities and Research grants (FaReBio di Qualità" project and the Flagship Project "NanoMAX").

Biophysical characterization of skeletal muscle-derived exosomes

Daniele P. Romancino[1], Samuele Raccosta[2], Valentina Buffa[1], Antonella Lavanco[1], Vincenzo Martorana[2] , Mauro Manno[2] and Antonella Bongiovanni[1]
[1]Institute of Biomedicine and Molecular Immunology (IBIM), National Research Council (C.N.R.), Palermo, Italy,
[2]Department of Genetics, St Jude CRH, Memphis, United States, [3]Institute of Biophysics (IBF), National Research Council (C.N.R.), Palermo, Italy

Consiglio Nazionale delle Ricerche

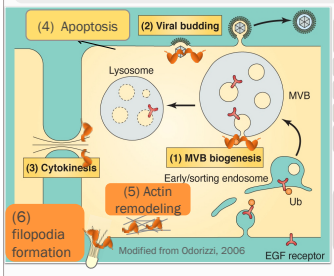
Background: Almost all living organisms on earth shed extracellular vesicles (EVs) into their microenvironment. Extracellular vesicles are spherical bilayered proteolipids with an average diameter of 20-1,000 nm, which are known to harbor a specific subset of bioactive molecules reflecting their originating cell types and conditions. Recent advances in this fast growing biology field have facilitated several insights: extracellular vesicles as mediators of multifaceted pathophysiological functions in intercellular communication, rich sources of biomarkers for noninvasive diagnosis and prognosis of various human diseases, and tools for diverse therapeutic approaches. Recently, we have shown that also skeletal muscle (SkM) cells can release Alix-positive exosomes, suggesting the importance of exosomes in skeletal muscle biology. Since skeletal muscle is the largest organ in the body and it is now considered a secretory organ, it can be expected that essential discoveries on SkM-derived exosomes in health, disease, and regeneration will provide an important link between genetic and epigenetic impact factors. Coupled to nanotechnology, engineered exosomes might be useful to allow the tissue regeneration, and to recover from muscle atrophy and/or injury.



Our goal is to understand how muscle cells generate these vesicles and what their regulators are. To this end, we used biophysical techniques (Dynamic Light Scattering, DLS, small-angle X-ray, SAXS, and Nanoparticle Tracking Analysis, NTA), and immunoblot analyses of exosome specific markers, after inhibition of protein lipidation.

Question 1. The multiple personalities of Alix.

Alix, a membrane bending protein involved in exosome biogenesis?



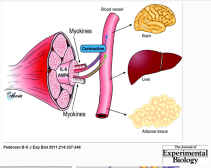
ALIX functions directly on membranes:
(1) MVB biogenesis
(2) viral budding
(3) cytokinesis
(6) filopodia formation

All related to negative mb curvature and 100-nm scale structures

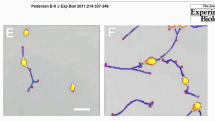
Alix contains a convex face containing a positively charged surface that might function as membrane bending domain to generate or scaffold a negative curvature within the membrane

Question 2. Muscle-derived exosomes are released through an immediate mode

Skeletal muscle as secretory organ, to support tissues homeostasis and regeneration?

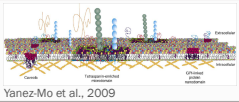


Skeletal muscle is the largest organ in the body and can account for the largest exosome fraction in body fluids.



The possible role of extracellular vesicles in the field of nerve regeneration is just beginning to be investigated: extracellular vesicles from muscle (the target end organ of motor neurons) have significant effects on motor neuron survival and neurite outgrowth (Madison et al., 2014).

Until now, myokines from muscle-cell secretome provided a conceptual basis to explain how muscles communicate to other organs. Rome's Lab demonstrated that exosome-like vesicle derived by myotubes may generate endocrine signals during myogenesis, inducing myoblast growth arrest and committed cells to differentiate.


Yanez-Mo et al., 2009

Question 3. Protein lipidation as possible post-translational modification (PTM) of specific regulators of exosomes biogenesis?

➢ *Palmitoylation may specifically contribute to the organization and flexibility of the tetraspanin-web (TEM)*

➢ The Intracellular Interactome of Tetraspanin-enriched Microdomains Reveals Their Function as Sorting Machineries toward Exosomes[*C]

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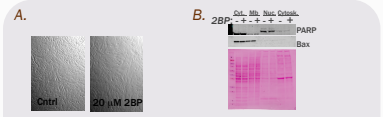
Palmitoylation Inhibitor in muscle-derived nanovesicles: **2-bromopalmitate (2BP), a non-metabolizable palmitate analog that blocks palmitate incorporation into proteins**

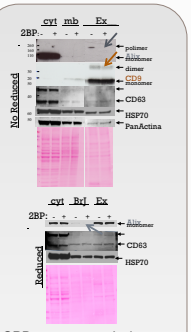


No morphological changes (*A.*), nor apoptotic signs (*B.*) following 2BP treatment (24 hrs)

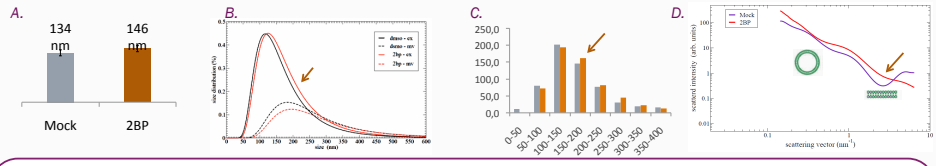
Mock-treated DIII C2C12: $6x10^8$ *vesicles/ml*

2BP-treated DIII C2C12: $9x10^9$ *vesicles/ml*

A. 2BP treatments induce a significant increase of the exosome number (DLS: 2x; NTA: 14x!) and *B.* of concentration of C2C12-derived exosomal proteins (BCA assays)



2BP treatment induce a significant reduction of Alix and CD9 polimers in exosomes, as well as a decrease of Alix monomer in the Brij010 soluble membrane fraction (mb domain enriched in tetraspanins)



A. Average exosome diameter significantly increase (9%) after 2BP treatments (media of 1xNTA and 3xDLS analyses). *B.* Representative DLS analysis. *C.* The 2BP treatments induce a different vesicles size distribution: an increase in number of vesicles with diameter of 150-200 nm (NTA analyses). *D.* Scattered intensity of SkM-derived exosomes by SAXS. The scattering intensity curve provides information about the size (low q) and bilayer thickness (high q) of the vesicles. The structural organization of the lipid bilayer of 2BP-treated exosomes (red line) is qualitatively different compared to non-treated myotube-derived exosomes (purple line). These data (repeated using different samples and beamlines) are suggestive of a loss of the structural order of the exosome membrane in the absence of palmitoylation. We can speculate that a laterally anisotropic structure on the vesicle surface, indicative of large protein clusters or membrane microdomain, disappeared following palmitoylation-inhibition; further analyses would address or confirm the nature of it.

Conclusion: Muscle exosomes could represent a previously undiscovered cellular communication system among muscle cells, which could play an important role in the maintenance of striated muscle homeostasis, regeneration, and repair. The biogenesis of SkM-derived exosomes differs from that of other mononucleated cells and might be related to Alix and its PTM (lipidation). Our results show that the inhibition of palmitoylation results in: i) increase of the number and diameter of exosomes, ii) modification of the exoxome membrane, and iii) significant decrease of polimeric forms of Alix and CD9 proteins. We propose that palmitoylation might function to regulate the proper function of SkM-derived exosome. *Future Plans*: The role of Alix in the biogenesis of and the heterogeneity of SkM-exosomes will be further confirmed and addressed by characterization of the Alix animal models (generated in d'Azzo lab), and biophysical means (DLS, FCS, and SAXS). Furthermore, we will analyze the function/s of muscle exosomes and palmitoylation in muscle differentiation and repair.

This work was supported by Italian Ministry of Economy and Finance and Ministry of Education, Universities and Research grants ("FaReBio di Qualità" project and the Flagship Project "NanoMAX".

Heterogeneity of skeletal muscle-derived extracellular nanovesicles and role of protein lipidation

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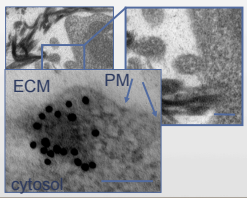
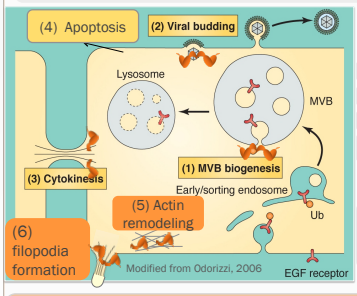
[2]Department of Genetics, St Jude CRH, Memphis, United States, [3]Institute of Biophysics (IBF), National Research Council (C.N.R.), Palermo, Italy

Background: Several cell types have the capacity to secrete nanometer-sized vesicles, exosomes, that contain cell-specific collections of proteins, lipids, and genetic material. Research on exosomes has been focusing primarily on the immune system and tumor cells; recently, we have shown that skeletal muscle (SkM) cells can release Alix-positive exosomes, suggesting the importance of exosomes in skeletal muscle biology. We believe that exosome biogenesis in muscle may differ with that of other mononucleated cells, being sustained by a direct budding of exosome from the plasma membrane. Since skeletal muscle is the largest organ in the body and it is now considered a secretory organ, it can be expected that essential discoveries on SkM-derived exosomes in health, disease, and regeneration will provide an important link between genetic and epigenetic impact factors.
Our goal is to understand how muscle cells generate these vesicles and what their regulators are. To this end, we used biophysical techniques (Dynamic Light Scattering, DLS, and Nanoparticle Tracking Analysis, NTA), and immunoblot analyses of exosome specific markers, after inhibition of protein lipidation



Question 1. The multiple personalities of Alix.
Alix, a membrane bending protein involved in exosome biogenesis?

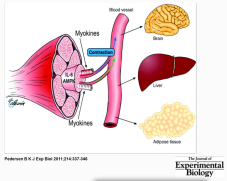


ALIX functions directly on membranes:
(1) MVB biogenesis
(2) viral budding
(3) cytokinesis
(6) filopodia formation

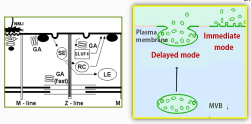
All related to negative mb curvature and 100-nm scale structures

Alix contains a convex face containing a positively charged surface that might function as membrane bending domain to generate or scaffold a negative curvature within the membrane

Question 2. Muscle-derived exosomes are released through an immediate mode
Skeletal muscle as secretory organ?



Skeletal muscle is the largest organ in the body and can account for the largest exosome fraction in body fluids.



The intracellular architecture of highly organized multinucleated skeletal muscle fibers differ from mononucleated cells. We propose, exosome biogenesis in muscle may differ with that of other mononucleated cells

Pedersen, 2019; Towler et al., 2004, Shen et al., 2011

Question 3. Protein lipidation as possible post-translational modification (PTM) of specific regulators of exosomes biogenesis?

- Palmitoylation may specifically contribute to the organization and flexibility of the tetraspanin-web
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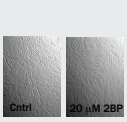

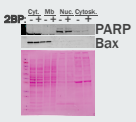

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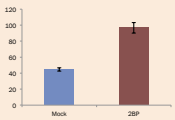

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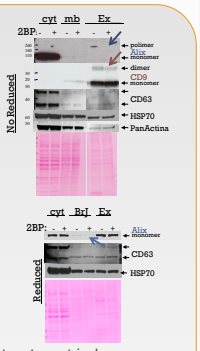

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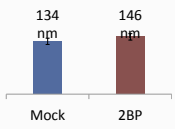

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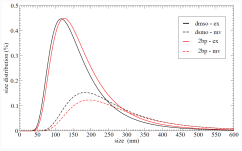

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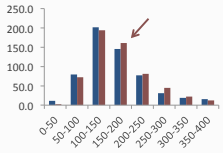

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