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Co-Mentor: Assoc. Prof. Mladenka Malenica

SVEUČILIŠTE U RIJECI

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Stanično specifične glikozilacije proteina CD63 otkrivene novoproizvedenim mišjim monoklonskim protutijelima

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1. Assoc. Prof. Elitza Markova Car

2. Asst. Prof. Ilija Brizić

3. Assoc. Prof. Mladenka Malenica

4. Assoc. Prof. Kristina Grabušić

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SUMMARY

CD63 is an evolutionary conserved transmembrane glycoprotein which is ubiquitously expressed in mammalian cells and abundantly located in late endosomes and lysosomes. It has no enzymatic activity, but exerts its functions by interacting with other proteins to act on a wide range of cellular processes like cytoskeletal organisation, cell adhesion and motility. At least some of these CD63 functions might explain the association of CD63 with cancer metastasis seen in clinical settings. However, for a deeper understanding of CD63 biology, the research community requires novel antibodies suitable for a systematic biochemical characterisation of CD63.

This study provides characterisation of two newly produced mouse monoclonal antibodies directed against mouse CD63: mCD63.07 and mCD63.08. Both antibodies bind to a linear epitope located at the extracellular portion of CD63 and are highly specific and sensitive in CD63 detection by immunoblot and immunofluorescence analyses based on signal comparison between wild type and CD63 knockout mouse fibroblasts. Moreover, the knockout validated antibodies were able to distinguish multiple CD63 glycoforms in several murine cell lines of different origins indicating that CD63 might have cell-type specific forms and functions. Finally, with the newly developed immunofluorescence protocol including protein denaturation, reduction and deglycosylation, novel antibodies detected significant CD63 amounts in cellular parts other than late endosomes and lysosomes.

Taken together, the newly developed antibodies might become a valuable tool to study CD63 biology thanks so the their two main features: ability to detect multiple CD63 glycoforms and to identify potentially novel cellular localisation of CD63.

Key words: tetraspanins, CD63 protein, glycosylation

SAŽETAK

Tetraspanin CD63 je ubikvitarno izražen protein u sisavaca koji se većinom nalazi u kasnim endosomima i lizosomima. CD63 nije enzimski aktivan, on svoje mnogobrojne funkcije izvršava preko interakcija s drugim proteinima te pritom sudjeluje u širokom rasponu staničnih procesa poput citoskeletne organizacije, stanične adhezije i pokretljivosti. U kliničkim je postavkama uspostavljena veza između CD63 i metastatskog potencijala tumora, za koju mogu biti odgovorne gore spomenute funkcije CD63. Međutim, za potpunije razumijevanje biologije CD63 proteina, istraživačkoj su zajednici potrebna nova protutijela pogodna za sustavnu biokemijsku karakterizaciju CD63 proteina.

Ovim su radom karakterizirana dva novoproizvedena mišja monoklonska protutijela usmjerena protiv mišjeg CD63: mCD63.07 i mCD63.08. Na temelju usporedbe signala između mišjih fibroblasta divljeg tipa i mišjih fibroblasta CD63 *knockout* tipa, utvrđeno je da se oba protutijela vežu na linearni epitop smješten na izvanstaničnom dijelu CD63 i da su vrlo specifična i osjetljiva u detekciji CD63 imunofluorescentnom i imunoblot metodom. Nadalje, *knockout* validirana protutijela mogu razlikovati višestruke glikoforme CD63 u nekoliko mišjih staničnih linija različitog podrijetla, što ukazuje da CD63 može imati stanično specifične forme i funkcije. Naposlijetku, s novorazvijenim protokolom imunofluorescencije, koji uključuje denaturaciju proteina, redukciju i deglikozilaciju, nova protutijela detektirala su značajne količine CD63 u odjeljcima stanice koji nisu kasni endosomi i lizosomi.

Na temelju dobivenih rezultata, novorazvijena antitijela mogla bi postati vrijedan alat za proučavanje biologije CD63 zahvaljujući njihovim dvjema glavnim značajkama: sposobnosti otkrivanja više glikoformi CD63 i identificiranja potencijalno nove stanične lokalizacije CD63.

Ključne riječi: tetraspanini, CD63 protein, glikozilacija

CONTENTS

1. INTRODUCTION

1.1. CD63 belongs to the tetraspanin family

Tetraspanins are membrane-spanning proteins involved in a large variety of biological processes such as adhesion, migration, differentiation, signalling, immune response, pathogen entry, tumour progression and metastasis (1). Tetraspanins are found in all multicellular organisms, but their number varies between species. For example, 20 members of the tetraspanin protein family were identified in *Caenorhabditis elegans*, 33 in *Homo sapiens* and 37 in *Drosophila melanogaster* (2). Tetraspanin research has been driven by interests in particular scientific fields such as cancer, immunology and extracellular vesicle research. Such targeted research and the fact that tetraspanins are markers of extracellular vesicles (discussed in section 1.1.4.) have led to several tetraspanins (CD63, CD81 and CD9) being researched the most.

Tetraspanins have no catalytic activity, they execute their function through organising molecules in the plasma membrane. This organisation facilitates cellular processes because all the components required for a particular process are gathered within a membrane compartment. These compartments are called tetraspanin-enriched microdomains (TEMs), and mainly contain different tetraspanins along with their binding partners (mostly integrins, members of the immunoglobulin superfamily, proteases and signalling receptors) (1). Each cell type contains specific molecules clustered in TEMs resulting in direct involvement of tetraspanins in a vast number of processes.

The ability of tetraspanins to associate with numerous partners along with tetraspanin redundancy, makes it difficult to study their molecular mechanisms. In addition, the overall tetraspanin structure protrudes above

the lipid bilayer by only 4–5 nm, making them often inaccessible to cell-surface labelling reagents (e.g. biotin) and complicating immunological detection (2).

1.1.1. Structural features of tetraspanins

A major feature of the tetraspanin family is the highly conserved protein structure consisting of four transmembrane domains (TMs), two extracellular loops and a short intracellular loop accompanied by N-terminal and C-terminal cytoplasmic tails (**Error! Reference source not found.**).

The TMs are highly conserved among tetraspanins. Even though several protein classes contain four TMs, they are not classified as tetraspanins unless they contain characteristic structural features: polar residues (Asn, Gln and Glu) within TM 1, 3 and 4; as well as four to eight conserved cysteine residues and a Cys-Cys-Gly (CCG) motif within the extracellular region (3). Moreover, intramolecular interactions between TMs are necessary for the proper synthesis and maturation of tetraspanins (4).

The extracellular domains (EC1 and EC2), also referred to as the small extracellular loop (SEL) and the large extracellular loop (LEL), are the least conserved tetraspanin regions. However, the EC2 region (70-140 amino acids, aa) contains crucial residues that are highly conserved among tetraspanins (4). All tetraspanins have three cysteine residues within EC2, two of which define the previously mentioned CCG motif located 28-47 residues after the third TM and a cysteine residue proximal to the fourth TM. The majority of tetraspanins (94%) contain a fourth cysteine in the Pro-Xaa-Xaa-Cys (PXXC) motif where Xaa represents any amino acid (4). The crystal structure of EC2 from CD81 confirmed the assumption that the four conserved cysteine residues form two intramolecular disulphide bonds, thus stabilizing the EC2 structure (5). Furthermore, the presence of two additional cysteines constituting a third disulphide bond is possible in many

tetraspanins, while only a small number of tetraspanins contains a total of eight cysteines creating four disulphide bonds (6).

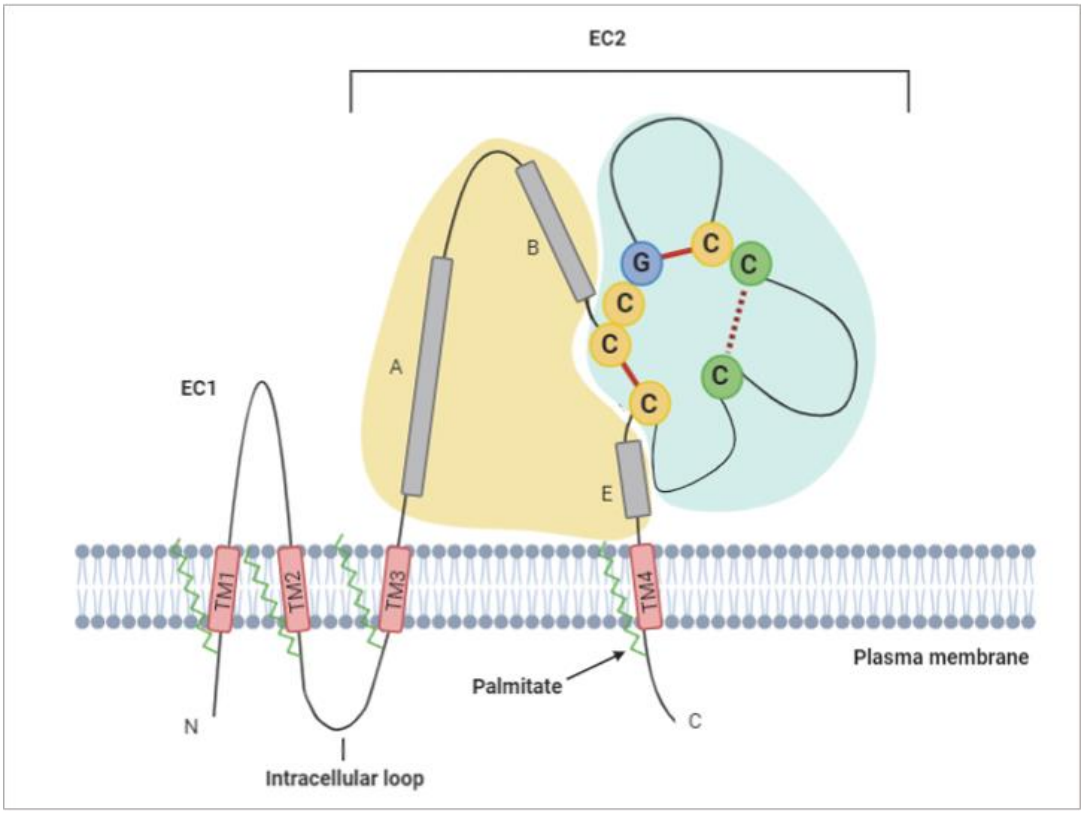

Figure 1. General features of tetraspanin proteins. *Tetraspanins comprise four transmembrane regions (TM1-4), two extracellular domains (EC1 and EC2), a small intracellular loop and short N- and C-terminal cytoplasmic tails. EC2 is composed of a constant part (highlighted in yellow) containing alpha helices (A, B and E) and variant part (highlighted in blue). The variant part always contains a CCG motif and two other conserved cysteines (C, yellow) which form disulphide bonds denoted by red lines. Most tetraspanins have two additional cysteines (green) which form another disulphide bond (dotted red line). Membraneproximal cysteines are susceptible to palmitoylation. The image was made in ©BioRender (biorender.com) based on Hemler et al (2).*

Additionally, the crystal structure of EC2 revealed that this region was composed of five α-helices, three (A, B, and E) located in the constant region and two (C and D) in the variable region (5). The constant region is considered to be responsible for tetraspanin homodimerisation via hydrophobic interactions while the variable is responsible for protein

interactions with non-tetraspanin proteins (4). When EC1 domain is absent or replaced, EC2 surface expression is reduced because the protein remains within the cell, therefore EC1 is assumed to mediate tetraspanin translocation to the cell surface (4).

Of the 200-350 amino acid residues that make up a tetraspanin, only 12-25 of them constitute the cytoplasmic domain. This domain consists of a very short intracellular loop (mainly 4 residues) and N-terminal and C-terminal tails (8-21 residues). Despite its small size, this domain is responsible for various intracellular protein interactions and subcellular localisation of tetraspanins (7).

1.1.2. Posttranslational modifications of tetraspanins

Posttranslational modifications of tetraspanins include glycosylation of the extracellular domain and palmitoylation in the intracellular regions. Both modifications regulate the ability of tetraspanins to associate with binding partners, thus regulating the formation of TEMs which are further described in the following section (6). Glycosylation of tetraspanins is achieved by the addition of N-glycans. The number and location of glycosylation sites vary among tetraspanins. Although glycosylation sites are mainly located in the EC2 region, they may be present in the EC1 region (CD9) or not present at all (CD81) (4), (8). Four intracellular cysteines, one proximal to each transmembrane domain, are evolutionarily conserved among tetraspanins and can be posttranslationally modified with the covalent addition of palmitate (Figure 1). Palmitoylation contributes to the creation of TEMs by promoting hydrophobic tetraspanin-tetraspanin interactions (6).

1.1.3. Tetraspanin interacting partners and enriched microdomains

Until recently, tetraspanins were thought to have no ligand binding sites, but the analysis of the entire CD81 crystallographic structure revealed that it contained an intramembrane cavity capable of binding cholesterol (5). The conducted computer simulations suggested that cholesterol binding induces a change in the conformation of CD81 (5). When bound to cholesterol, CD81 is in a closed conformation that is less likely to interact with partner proteins. The binding of cholesterol is not a cell-surface receptor–ligand binding interaction as the extracellular tetraspanin domains block the binding of extracellular cholesterol. The bound cholesterol most likely comes from the plasma membrane. Regulation of tetraspanin conformation by cholesterol may be one of the ways how protein interactions in TEMs are modulated (5).

The C-terminal tail of some tetraspanins contains a tyrosine-sorting motif Tyr-Xaa-Xaa-Φ (YXXΦ), where X is any amino acid and Φ represents a bulky hydrophobic amino acid. YXXΦ promotes protein localisation into intracellular compartments (described in more detail in section 1.2.2.). There is a widespread distribution of all tetraspanins in the endosomal system and extracellular vesicles, but only 12 tetraspanins have the tyrosine-sorting motif. Therefore, it is likely that the localisation of a tetraspanin without this motif occurs via interactions with those who have it. Furthermore, co-immunoprecipitating studies have shown that cytoplasmic domains are involved in protein interactions with phosphatidylinositol-4 kinase, protein kinase C, G protein, GTPase Rac, erzin and other proteins (1).

Tetraspanins participate in numerous physiological processes as molecular organizers. By facilitating grouping of their binding partners in the plasma membrane, tetraspanins create functional complexes in TEMs (**Figure 2**). Several types of molecular interactions, described on the basis of detergent resistance, influence the formation of TEMs (3). The first level

includes interactions between tetraspanins and their specific partners as well as the homophilic tetraspanin-tetraspanin interactions. These primary interactions are direct, strong and resistant to harsh detergents. The next level involves indirect, weaker secondary interactions that form between two different tetraspanins. These heterophilic tetraspanin-tetraspanin interactions bring primary complexes closer, assembling a network of secondary interactions. The final level consists of very weak interactions among palmitate chains attached to tetraspanins, which stabilise secondary complexes.

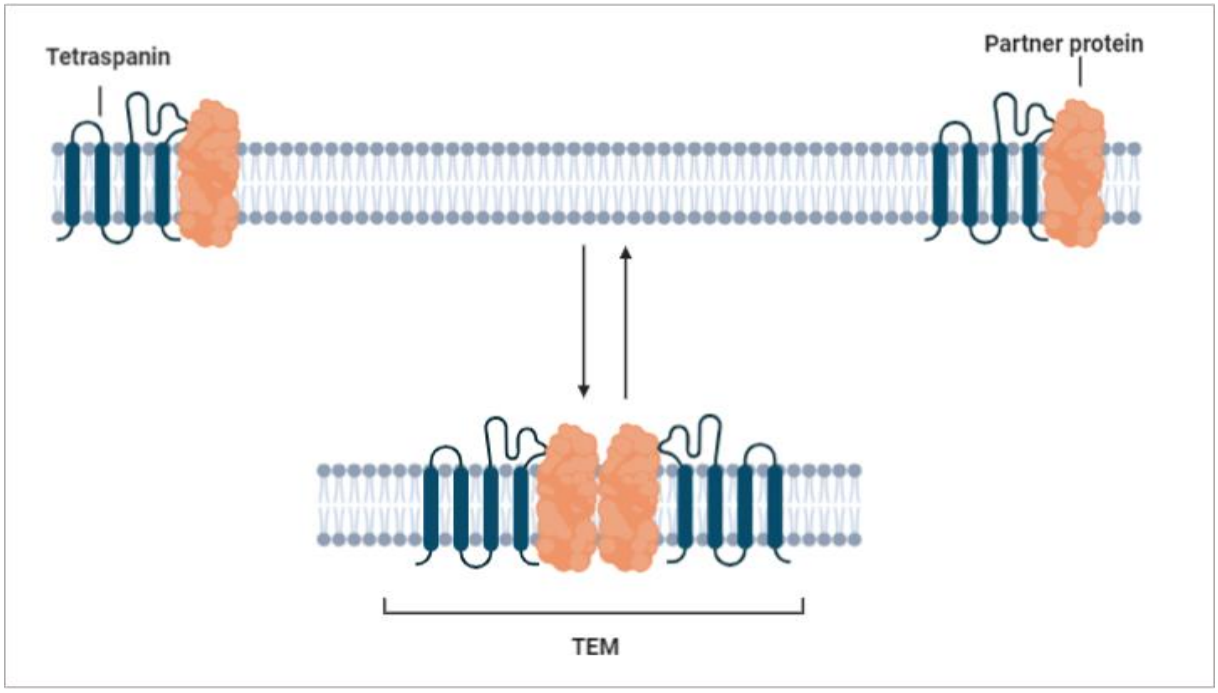

Figure 2. An example of a tetraspanin enriched microdomain (TEM). *TEMs compartmentalise a cellular process by bringing together a set of components. The image was made in ©BioRender (biorender.com).*

Treatment of cells with anti-tetraspanin antibodies results in physiological and morphological cell changes, implying a major functional significance of tetraspanins. Therefore, knockout mice were expected to have considerably disrupted phenotypes. However, the phenotype changes

were mild and did not meet the predictions based on results of antibody testing (9). The reason for this inconsistency is that antibodies affect individual tetraspanins, but also interfere with their associated binding partners within TEMs, while the deletion of one tetraspanin leaves TEMs intact. In addition, redundancy of tetraspanin functions and compensatory effects contribute to mild phenotype changes and complicate the analysis of tetraspanins by loss-of-function studies.

1.1.4. Tetraspanins are secreted as constituents of extracellular vesicles

Extracellular vesicles (EVs) are circular nanoparticles secreted by most cell types when multivesicular bodies (MVBs) fuse with the cell membrane (described in section 1.2.2.). EVs represent a way of intercellular communication because they can transport proteins, lipids and nucleic acids between cells. It is possible for EVs to influence the recipient cell based on the information they carry. Different types of EVs are classified based on their biogenesis, cargo, density and size. Since EVs are secreted outside the cell, it is possible to isolate them from all body fluids, which offers a potential use of EVs as non-invasive biomarkers, and in therapeutic purposes. To elucidate the potential of EVs, we need a far better knowledge of their biology. Tetraspanins are valuable tools in the study of EV biology because EVs are highly enriched in tetraspanins (from 10- to 100-fold compared to their content in the parent cells), especially CD9, CD37, CD63, CD81 and CD82 which are used as EV biomarkers (3). Furthermore, tetraspanins have a potential role in all aspects of EV biology which is why it is essential to develop approaches that could target tetraspanins and enable the clinical use of EVs.

1.2. CD63 is one of the first discovered tetraspanins

CD63 is one of the first discovered tetraspanins, it was initially detected over thirty years ago on the surface of early stage human melanoma cells and, shortly after, on the surface of activated platelets. Further studies discovered that most CD63 colocalised with lysosome-associated membrane proteins LAMP-1 and LAMP-2. The colocalisation, along with other similarities (YXXΦ motif in the C-terminal cytoplasmic tail, highly glycosylated protein) led to the wrong classification of CD63 as LAMP-3. Sequence homology studies later showed that CD63 belonged to a completely new protein family of tetraspanins.

1.2.1. Biochemical properties of CD63

CD63 is a ubiquitously expressed protein located in the endosomal system and to a lesser extent on the plasma membrane (Figure 3). Most tetraspanins localise to the plasma membrane, but CD63 is one of the few tetraspanins that mainly localise intracellularly.

Based on the primary protein sequence of CD63, its molecular weight was predicted 25 kDa, this weight represents the protein core with no posttranslational modifications (10). Glycosylation is a common posttranslational modification which involves the covalent binding of a carbohydrate group to a protein. Oligosaccharides are bound either to the nitrogen atom in the side chain of asparagine (N-linked) or to the oxygen atom in the side chain of serine or threonine (O-linked). N-linkage of oligosaccharides occurs at specific asparagine residues that are a part of the Asn-X-Ser/Thr sequence, where X is any amino acid residue other than proline. Therefore, potential glycosylation sites can be predicted from the primary protein sequence. However, not all potential sites are glycosylated. The Asn-X-Ser/Thr sequence is required, but not sufficient for N-glycosylation to occur, as this depends on other protein characteristics and on the cell type in which the protein is synthesised. According to the UniProt

database, mouse CD63 protein contains four possible N-glycosylation sites in the EC2 domain. Glycosylation significantly increases the molecular weight of CD63 (10). Additionally, CD63 has two disulphide bonds in the EC2 domain.

Palmitoylation is a a posttranslational modification that includes the covalent binding of palmitic acid to intracellular cysteine residues of membrane proteins. CD63 palmitoylation was studied in platelets where it was palmitoylated at a low level in unstimulated platelets, but at a higher level in trombin activated platelets. The palmitoylation of CD63 in activated platelets contributed to its interaction with CD9 in the plasma membrane (11).

1.2.2. Subcellular localisation and trafficking of CD63

Next to the previously described biochemical properties, CD63 protein contains a tyrosine-sorting motif (YXXΦ) responsible for clatrin-mediated endocytosis of CD63 at the plasma membrane and the subcellular localisation of CD63 in late endosomes and lysosomes (7). In endocytosis, the material to be drawn into the cell is surrounded by a region of the plasma membrane, which then buds off inside the cell to form a endocytic vesicle (12). CD63 is internalized via receptor-mediated endocytosis in clathrin-coated vesicles and may also be endocytosed via caveolae. Formed vesicles merge with early endosomes from which the vesicle content either recycles back to the plasma membrane or becomes incorporated into intraluminal vesicles (ILVs) (7). Early endosomes mature into late endosomes, also known as MVBs. During maturation, a large number of ILVs are formed through invagination of the limiting endosomal membrane that buds into the endosomal lumen. ILVs can be degraded when MVBs fuse with or mature into lysosomes, or released into the extracellular space by fusion of MVBs with the plasma membrane. Secreted ILVs are called EVs. Within the endosomal system, CD63 is enriched in ILVs/EVs and lysosomes (7).

Furthermore, CD63 is present in lysosome-related compartments called secretory lysosomes. These include the α-granules of megakaryocytes, dense and α-granules of platelets, cytotoxic granules of T-cells, crystalloid granules of eosinophils, secretory granules of basophilic granulocytes and mast cells, Weibel-Palade bodies of endothelial cells and a subset of pre-melanosomes in melanocytes (9). Upon stimulation, secretory lysosomes fuse with the plasma membrane, releasing their secretory product and transferring CD63 to the cell surface, resulting in increased surface expression of CD63.

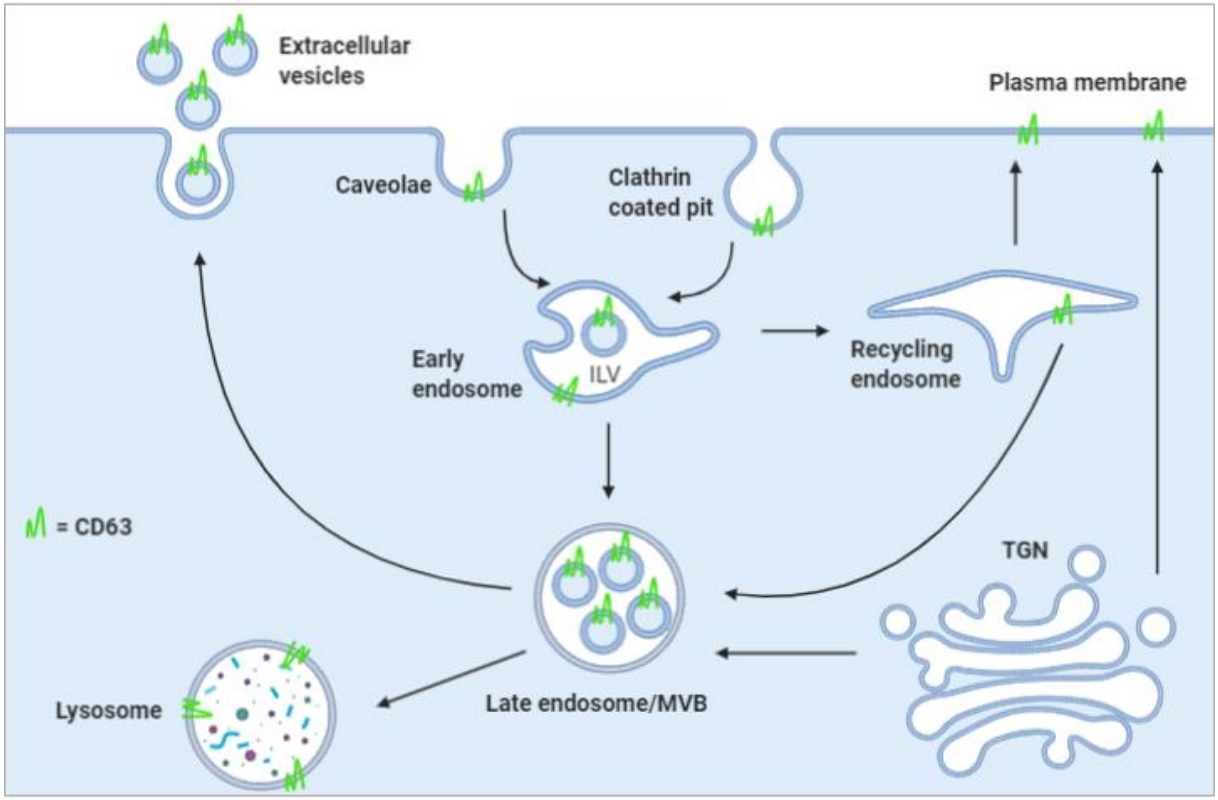

Figure 3. CD63 trafficking. *CD63 circulates in cell by endocytotic routes starting from plasma membrane via early and late endosomes and is finally enriched in lysosomes. CD63 gets incorporated into intraluminal vesicles (ILV) accumulated in multivesicular bodies (MVB) which fuse with plasma membrane and are released as extracellular vesicles outside the cell (7). From the trans-Golgi network (TNG) CD63 is either transported to the plasma membrane or directed to the multivesicular body (MVB). The image was made in ©BioRender (biorender.com) based on Pols et al (7).*

The trans-Golgi network (TGN) functions as a sorting station that directs newly synthesised or recycled glycoproteins to various subcellular locations. Depending on their signal sequence, proteins are sorted into different transport vesicles destined to lysosomes, secretory vesicles, or the plasma membrane. Lysosomal membrane proteins travel from the TGN to lysosomes either directly through transport vesicles that fuse with the MVBs, or indirectly, via the plasma membrane and subsequent endocytosis.

The sorting of lysosomal membrane proteins, such as LAMP-1, LAMP-2 and CD63 depends on tyrosine and dileucine-based sorting signals positioned within the C-terminal cytosolic tail. The YXXΦ motif is a tyrosine-based sorting signal required for clatrin-mediated endocytosis at the plasma membrane (indirect pathway), but is also connected to direct TGN-to-lysosome targeting. Trafficking of LAMPs and CD63 is differentially regulated even though they both contain the YXXΦ motif. LAMP-1 and LAMP-2 are delivered to lysosomes mainly via the direct route while CD63 can take the direct and indirect pathway. The CD63 YXXΦ motif has additional characteristics responsible for the indirect TGN-to-lysosome targeting. The Gly-Tyr-Glu-Val-Met (GYEVM) sequence of CD63 has a glycine residue before the critical tyrosine and the entire sequence is located 6-9 residues from the transmembrane domain.

The GYEVM sequence of CD63 is important for the binding of adaptor proteins (APs) responsible for trafficking of molecules. AP-2 is involved in clathrin-mediated endocytosis of cell surface CD63. After fusion of the clathrin-coated vesicles with early endosomes, CD63 is either transported to MVBs or recycling endosomes (Figure 3). From recycling endosomes CD63 can be recycled back to the cell surface or transported to MVBs by associating with AP-3. Mutations in the GYEVM sequence cause CD63 to lose its intracellular localisation due to lack of binding to AP-2 and AP-3, resulting in a increased cell surface expression (13).

1.2.3. Physiological roles of CD63 in different cells

As most tetraspanins, CD63 participates in an array of biological processes. The physiological roles of the CD63 protein vary depending on the cell type. In cells containing secretory lysosomes, such as basophiles, CD63 can be used as a cell activation marker. Activated cells secrete their contents by fusing secretory lysosomes with the plasma membrane, thereby significantly increasing the surface expression of CD63 (14). Several studies show that the involvement of CD63 in a wide range of processes is the result of it controlling the trafficking of partner proteins. As described in section 1.2.2., due to its tyrosine-based internalization motif, CD63 cycles between endosomes, lysosomes, secretory lysosomes and the plasma membrane. This enables CD63 to induce internalization of partner proteins from the cell surface and target them to MBVs, or secretory lysosomes. In gastric cells, CD63 regulates the trafficking of the heterodimeric ion pump H, K-ATPase between the cell surface and intracellular compartments (15). In kidney cells, CD63 regulates membrane-type 1 matrix metalloproteinase surface expression by targeting it to lysosomes, while in macrophages it targets synaptogamin 7 to lysosomes (16), (17). In T-lymphocytes, CD63 suppresses the surface expression of the C-X-C chemokine receptor type 4 (CXCR4) by directing CXCR4 to late endocytic compartments, rather than the plasma membrane (18).

CD63 plays a role in bone marrow-derived mast cell degranulation and tumor necrosis factor (TNF)-α secretion in response to FceRI stimulation, but not in response to other stimuli (19). In endothelial cells, CD63 acts as a P-selectin cofactor, thereby participating in the recruitment of leukocytes and platelets onto endothelial cells (20). Also, it regulates the internalization of vascular endothelial growth factor receptor 2 (VEGFR2) in response to VEGF signaling and participates in adhesion and migration of endothelial cells by promoting (VEGFR2)-β1 integrin complex formation and

downstream signalling (21). Finally, in neural stem cells, CD63 acts as a cell surface receptor for tissue inhibitor of metalloproteinase-1 and activates cell signaling cascades which result in cytoskeletal reorganization and cell migration (22).

1.2.4. Involvement of CD63 in tumour and other pathological conditions

In general, downregulation of CD63 expression is associated with elevated malignant potential in several types of tumour. The CD63 antigen was first discovered on early stage human melanoma cells, but as the melanoma progressed and the cells became more invasive, CD63 expression was reduced (23). *In vivo*, a negative correlation between CD63 expression and tumour invasiveness was found for astrocytoma, early-stage lung adenocarcinoma, esophageal, ovarian, breast and non-small cell lung cancer (24), (25), (26), (27), (28), (29). Taken together, these studies show that the lack of CD63 promotes cell motility and extracellular matrix degradation which increases the metastatic ability of cancer cells. The possible explanation for the suppresive role of CD63 is that CD63, along with other tetraspanins in TEMs, stabilises integrins that bind to the extracellular matrix (1). Also, CD63 regulates the endocytosis of proteins involved in tumour progression, such as membrane-type 1 matrix metalloproteinase (16).

CD63 is enriched in ILV membranes. ILVs found in MVBs can be broken down, released as exosomes, or incorporated into secretory lysosomes. The fate of proteins in endosomal compartments depends on the way they are incorporated into ILVs. Ubiquitinated proteins are recognized by the endosomal sorting complex required for transport (ESCRT), the ESCRT-dependent protein incorporation results in ILV degradation. The sorting of CD63 into ILVs is independent of ESCRT, however, this pathway is vaguely understood. Some authors proposed that

CD63 could be a functional part of the ESCRT-independent ILV formation, responsible for the intracellular transport of its binding partners. The data supporting this claim is the deletion of CD63 in melanocytes which results in ESCRT-dependent degradation of the premelanosome protein (PMEL), indicating that CD63 protects secretory lysosome cargoes from ESCRT-dependent degradation (7), (30). Furthermore, CD63 is involved in the proper targeting of neutrophil elastase to neutrophil secretory lysosomes and CD63 knockout mice showed altered secretory lysosomes (31), (9). Moreover, CD63 is deficient in patients with Hermansky-Pudlak syndrome (32). This syndrome is characterised by defects in melanosomes, platelet dense granules and lysosomes.

2. THE GOAL OF THESIS

Tetraspanin CD63 participates in a range of cellular functions like cell survival, cytoskeleton organisation, cell adhesion and migration. Although the large extracellular domain (EC2) of CD63 is known to be highly glycosylated, little is known about the role and regulation of CD63 glycosylation in various cellular functions due to lack of suitable antibodies. To address this issue, two new murine monoclonal antibodies targeting the EC2 domain of CD63 were produced at the Center for Proteomics at the School of Medicine in Rijeka.

The specific goals of this thesis were:

1. to determine antibody specificity on western blot by comparing protein lysates from wild type and CD63 knockout primary embryonic fibroblasts;
2. to analyse CD63 protein expression in murine microglia, mastocytoma, dendritic, macrophage, endothelial and fibroblast cell lines by western blot;
3. to determine whether the different protein bands detected on western blot are glycoforms of the CD63 protein;
4. to examine the usability of the antibodies on immunofluorescence and optimise the immunofluorescence protocol if needed;
5. to determine the specificity of antibodies on immunofluorescence using wild type and CD63 knockout primary embryonic fibroblasts;
6. to find the optimal antibody concentration for use on immunofluorescence.

3. MATERIALS AND METHODS

3.1. Primary mouse cells and cell lines

All cells were obtained at the University of Rijeka, Faculty of Medicine. Primary mouse embryonic fibroblasts of wild type (wt-MEF) and CD63 knockout mice (ko-MEF) were produced according to current ethical standards at the Department of Physiology, Immunology and Pathophysiology. Macrophage (RAW 264.7), dendritic (DC2.4) and endothelial cell lines (SVEC4-10) were provided by the courtesy of the Department of Histology and Embryology. Microglia (BV2), mastocytoma (P815) and fibroblast (B12) cell lines were provided by the courtesy of the Department of Physiology, Immunology and Pathophysiology.

Cells were cultured in Dulbecco's Modified Eagle Medium (Pan-Biotech) supplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all Pan-Biotech) at 37 °C and 5% CO_2.

3.2. Western blot

Cells were trypsinised, washed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4) and pelleted by centrifugation at 600 x *g* for 5 minutes at +4 °C. The supernatant was discarded.

Proteins were extracted by adding an appropriate volume of lysis buffer (RIPA buffer, Thermo Scientific + cOmplete™ Protease Inhibitor Cocktail, Roche, with addition of NaF and Na_3VO_4 to final concentrations of 10 mM and 5 mM, respectively) to the cell pellet and incubating it for 20

minutes on ice with occasional shaking. The mixture was centrifuged at 16 000 × *g* for 15 minutes at +4 °C to pellet the cell debris.

The pellet was discarded and the protein-containing supernatant was transferred to a new tube. Protein concentration was determined by the Bradford protein assay. Briefly, 2 µl of the supernatant was added to 1 ml of diluted Bio-Rad Protein Assay Dye Reagent Concentrate (1:5 in H_2O) and the absorbance of the sample was read at a wavelength of 595 nm with a Beckman Coulter DU730 UV/Vis Spectrophotometer. Protein concentration was calculated from the standard curve based on bovine serum albumin of known concentration. The protein-containing supernatant was either stored at -80 °C or immediately prepared for western blot (WB) analysis.

Protein samples containing 10 µg were prepared for WB analysis by adding 5x Laemmli buffer (1M Tris-HCl pH 6.8, 50% glycerol (v/v), 10% SDS (w/v), 0.05% bromophenol blue (w/v) and 10% 2-mercaptoethanol (v/v)) to make up one-fifth of the total sample volume. Prepared samples were heated for 10 minutes at 95 °C followed by short centrifugation.

Proteins were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 1x Running buffer (25 mM Tris, 192 mM Glycine and 0.1% SDS, pH 8.3) on 90 V to 130 V. A protein standard was used to monitor protein size (PageRuler™ Prestained Protein Ladder, Thermo Scientific).

After electrophoresis, proteins were transferred to a PVDF-P membrane (Millipore) at a constant voltage of 70 V for an hour and a half in a 1x Transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol). Following the protein transfer, the PVDF-P membrane was incubated with 5% milk in Tris-buffer saline (TBS, 20 mM Tris and 150 mM NaCl) for 15 minutes to block unspecific antibody binding. The membrane was incubated overnight at +4 °C on a shaker with mouse monoclonal primary antibody α-actin (1:80 000 in 5% milk in TBS, pan-actin, Millipore) or purified

mCD63.07 (lot001, 7µg/ml in 5% milk in TBS, University of Rijeka, Center for Proteomics).

The membrane was washed three times for 5 minutes with 0.05% Tween 20 in TBS (TBS-T) followed by incubation with the secondary anti-mouse antibody (1:25 000 in 5% milk in TBS, Jackson) for 30 minutes at room temperature. Afterwards, the membrane was washed three times for 5 minutes with 0.05% TBS-T. Blots were visualized using SignalFire™ ECL (Cell Signaling) and detected with the Alliance 4.7 (Uvitec Cambrige) imaging system.

3.3. PNG-ase F treatment of proteins

Whole cell protein extracts were treated with peptide-*N*-glycosidase F (PNG-ase F, New England Biolabs #P0704) according to manufacturer's instructions. Briefly, for each reaction, 2 µg of protein extract from wt-MEF, SVEC4-10 and P815 cells, or 15 µg of protein extract from B12, BV2, DC2.4 and RAW264.7 cells were mixed with 1 µl of Glycoprotein Denaturing Buffer (10x) and H_2O to give a total reaction volume of 10 µl. Proteins were denatured by heating the mixtures at 100 °C for 10 minutes. Next, a PNG-ase master mix was prepared by combining GlycoBuffer 2, 10% NP-40, H_2O and PNG-ase. A final reaction volume of 20 µl was made by adding 10 µl of the master mix to each reaction with denatured proteins. The enzymatic reaction was carried out at 37 °C for 1 hour. After deglycosylation, protein samples were prepared for WB analysis as previously described.

3.4. Antigen retrieval and PNG-ase F treatment of cells

For PNG-ase F treatment of cells a modified protocol was developed and applied based on published protocol (33). Briefly, cells were seeded into 24-well plates on 12 mm round coverslips and cultured for 24 hours. Cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. After fixation cells were washed with PBS. Protein denaturation and reduction was accomplished by treating the cells with an antigen retrieval (AR) mixture (6 M urea in 0.1 M Tris, pH 8.8 + 1:5 mercaptoethanol) at 95 °C for 30 minutes.

After AR, cells were washed three times with PBS and permeabilized for 7 minutes at room temperature with 0.5% Triton X-100 followed by washing in PBS. Coverslips with cells were treated by PNG-ase F (New England Biolabs) by using 500 units of PNG-ase F in GlycoBuffer 2. Coverslips were incubated at 37 °C for 2 hours. After PNG-ase F treatment, a standard protocol for immunofluorescence was used, starting with blocking as described below.

3.5. Immunofluorescence

Cells were seeded into 24-well plates on 12 mm round coverslips and cultured for 24 hours. After fixation with 4% PFA for 20 minutes at room temperature cells were washed with PBS and permeabilized for 7 minutes at room temperature with 0.5% Triton X-100. Unspecific binding was blocked with 5% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature. Cells were then incubated overnight at 4 °C with purified mouse mCD63.07 (lot002, 2-18 µg/ml) and mCD63.08 (lot003, 1-20 µg/ml) (both antibodies produced at the University of Rijeka, Center for Proteomics) or rat anti-mouse CD63 (1:200, MBL) in PBS with 0.1% Tween 20 and 5% BSA.

The next day, cells were washed three times with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (1:1000, Invitrogen) or Alexa Fluor 488 goat anti-rat IgG (1:1000, Invitrogen) secondary antibody in PBS with 0.1% Tween 20 and 5% BSA for 30 minutes in the dark at room temperature. The same secondary antibody was used for both primary antibodies because mCD63.07 is IgG2b isotype, while mCD63.08 is IgG2c isotype.

For nuclei visualization, cells were treated with DAPI (Sigma-Aldrich, 0.25 ng/ml) for 1 minute in the dark, at room temperature, followed by four washes with H_2O. Samples were mounted on a glass slide with Mowiol 4-88 (Hoechst AG) and left overnight at 4 °C before they were analysed by fluorescent (Olympus BX51) or confocal microscopy (Olympus FV3000).

3.6. Quantification of immunofluorescent signal

Images from the fluorescent microscope were quantified with the ImageJ program (National Institutes of Health, U.S.). For each analysed cell, the programme was set to provide the Area, Mean and Integrated density values. For each image, an area without any signal was measured, this area represented the Background noise of the image. After the signal of 100 cells per coverslip was measured, the Background readings were averaged to get the average Background noise of the coverslip. The last step was to calculate the corrected total cell fluorescence (CTCF) for each cell using the following formula: CTCF = Integrated density – (Area x average Background noise). After the CTCF of 100 cells per coverslip was calculated, the CTCF values were averaged and the standard deviation of the averaged CTCF value was calculated.

4. RESULTS

4.1. The newly produced mouse monoclonal mCD63.07 antibody specifically detects CD63 protein *in vitro*

Initial characterisation of antibody usability in different methods conducted during the production of hybridoma cell lines has shown that the newly produced mouse monoclonal mCD63.07 antibody provides a positive signal in western blot analysis. To test the antibody specificity, mCD63.07 signal in immublot analysis was first compared between primary mouse embryonic fibroblasts of wild type (wt-MEF) and CD63 knockout mice (ko-MEF) (Figure 4).

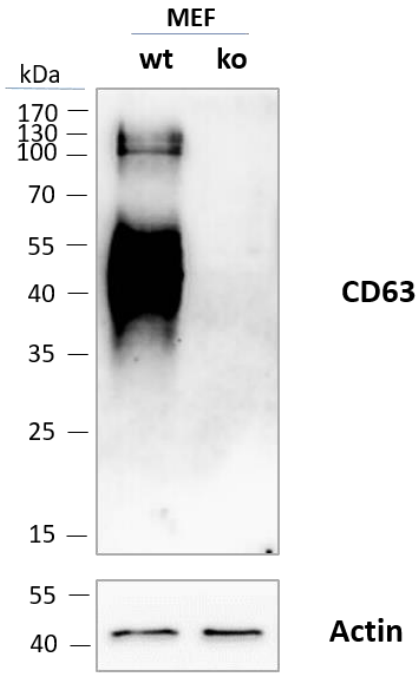

Figure 3. mCD63.07 antibody specifically detects CD63*. Total protein lysates from wild type (wt) and CD63 knockout (ko) primary mouse embryonic fibroblasts (MEF) were separated by SDS-PAGE and analysed by western blot. Immunodetection was performed with purified anti-CD63 mCD63.07 antibody and anti-actin. Signal was detected by chemiluminescence. The numbers on the left indicate the positions of protein marker bands. Shown is representative image of 3 independent experiments.*

Analysis of whole cell protein extracts from wt-MEF by mCD63.07 resulted in the detection of two discrete groups of bands: one group of bands starting above 35 kDa and continuing to slightly above 55 kDa, and another group of signals above 100 kDa. There was no signal detected by mCD63.07 in whole cell protein extracts of ko-MEF proving that the mCD63.07 specifically detects multiple forms of CD63 proteins in primary wt-MEF. Amounts of loaded proteins from both wt-MEF and ko-MEF were similar as shown by comparable actin levels.

4.2. CD63 protein level and forms are cell-type specific

After validation of mCD63.07 specificity in western blot analysis by comparing primary wt-MEF and ko-MEF, the analysis was expanded to cell lines of various cellular origin to determine if cell type affects CD63 protein expression level and forms. Whole cell protein extracts from fibroblast (B12), endothelial (SVEC4-10), microglia (BV2), macrophage (RAW 264.7), dendritic (DC2.4) and mastocytoma (P815) cell lines were separated by SDS-PAGE electrophoresis and immunodetected by the mCD63.07 and commercial anti-actin antibody (Figure 5). While actin was detected in comparable amounts, CD63 protein levels largely differed between analysed cell lines. The strongest CD63 signal was detected in P815, followed by lower, but still easily detectable levels in SVEC4-10 and B12 cells, and low levels in BV2 and DC2.4 cells which were clearly detected only in longer expositions (Figure 5c). RAW 264.7 was the only cell line in which no CD63 protein could be detected.

Next to the difference in CD63 protein level, the cell lines also showed diversity in sizes of detected bands. The CD63 signal range was the largest in P815 spanning from approximately 30-170 kDa. B12, SVEC4-10 and BV2 had a common span of signals at about 40-70 kDa and at around 100-170 kDa.

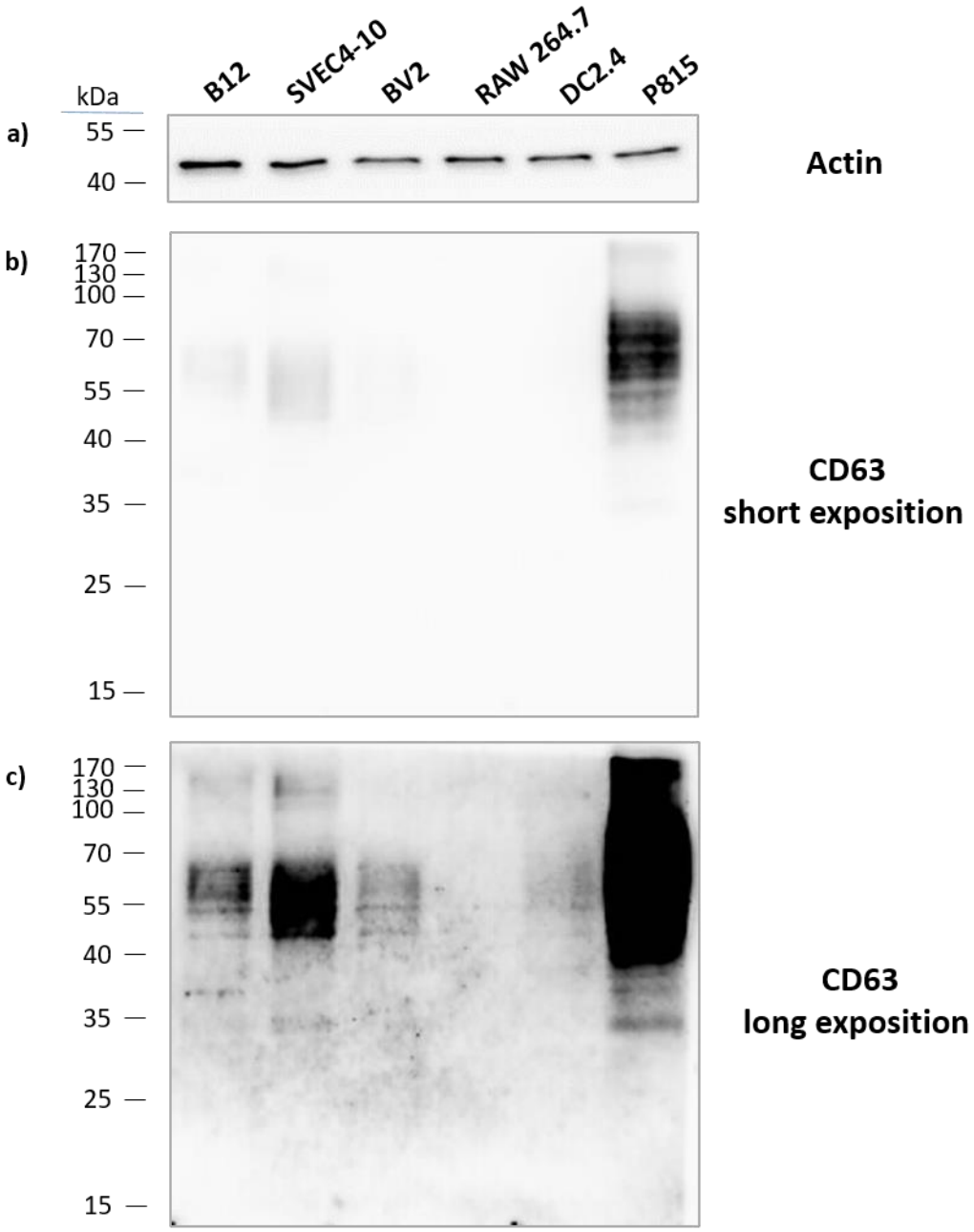

Figure 4. CD63 is expressed in various protein forms and levels depending on cell type. *Total protein extracts from six cell lines were separated by SDS-PAGE followed by immunodetection with anti-actin antibody as a loading control* ***(a)*** *and mCD63.07 for which chemiluminescent signal was recorded in a short (22 sec)* ***(b)*** *and a long (2 min)* ***(c)*** *exposition. Positions of protein marker bands are denoted on the left.*

To study cell-type specific forms of CD63 protein in more details, an additional western blot analysis was performed in which better protein separation was achieved by longer performed SDS-PAGE electrophoresis where all protein bands under 35 kDa were released from the gel. Additionally, to compensate for low level expression of CD63 in some cells and thus to enable detection and comparison of all CD63 forms, protein amount was accordingly increased for BV2 and DC2.4 cells (Figure 6).

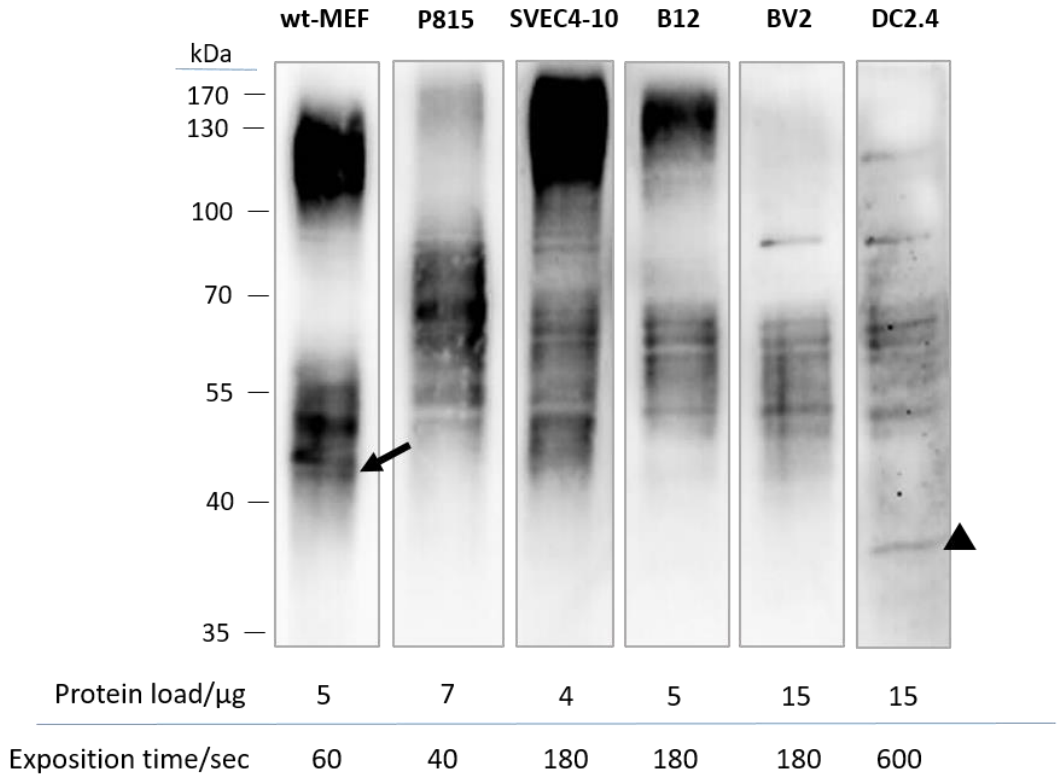

Figure 5. Cells can express specific CD63 protein forms. *Whole cell protein extracts of indicated cells were loaded in amounts relative to CD63 level expression and separated by SDS-PAGE followed by immunoblot detection with mCD63.07. Exposition time and protein load for each cell line are denoted on the bottom of the image. The arrow and triangle represent bands characteristic of individual cell lines. The numbers on the left indicate the positions of protein marker bands. Shown is representative image of 3 independent experiments.*

Thanks to the adapted amount of loaded proteins and better resolution achieved by releasing all protein bands under 35 kDa during SDS-PAGE separation, all analysed cells except wt-MEF demonstrated a better resolution of numerous distinguishable bands mostly in the range between roughly 55 kDa to 70 kDa. However, despite the presence of diverse bands it was not possible to pinpoint among them a potential unique band for certain cell line. Likewise, signals in B12, SVEC4-10 and P815 at position above 100 kDa contained several bands, but the resolution was not sufficient to detect potentially unique bands. However, wt-MEF contained a group of signals between 40 kDa and 55 kDa of which some might be unique among compared cells. Comparably to wt-MEF, DC2.4 also showed a potentially unique CD63 form whereby this signal appeared below 40 kDa and it was clearly separated from other bands.

4.3. The majority of CD63 posttranslational modifications are N-glycosylations

N-glycosylation is a common posttranslational modification of the CD63 protein that involves the covalent binding of a carbohydrate group to the nitrogen atom in the side chain of asparagine (N-linked carbohydrate). To see if the multiple protein bands shown in previous figures are indeed CD63 glycoforms, protein lysates were treated with PNG-ase F to cleave N-linked sugar residues and analysed by immunoblot (Figure 7). Based on different CD63 levels previously detected (Figure 5 and 6) in analysed cell lines, PNG-ase F treatment was performed on 2 µg i.e. 15 µg of protein extract from cells with high (wt-MEF, SVEC4-10, P815) i.e. low CD63 protein level (DC2.4, B12, RAW 264.7 and B12).

In PNGase-F untreated protein samples of wt-MEF, SVEC4-10, P815, BV2 and B12, CD63 was detected in immunoblot by mCD63.07 antibody as a collection of distinctive ranges of bands which were comparable to

previous results here described. Also consistent with previous results, ko-MEF and RAW 264.7 showed no CD63 signal without PNG-ase F treatment. Likewise, no CD63 could be detected in DC2.4 cells in PNG-ase F untreated samples, although previous analysis on larger amount of proteins (see Figures 5 and 6 for comparison) could detect low levels of CD63 protein relative to other analysed cells.

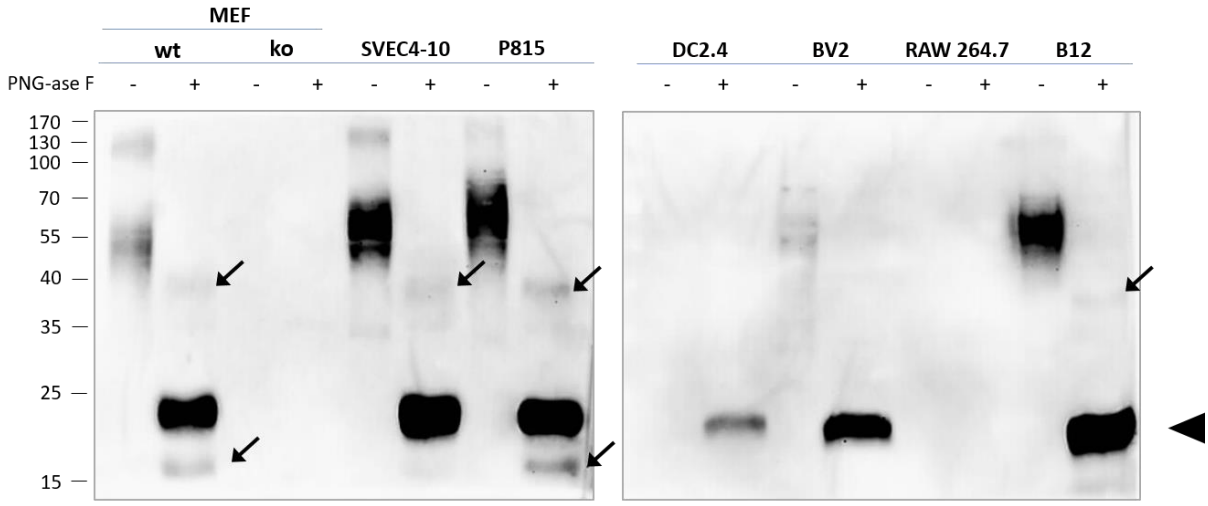

Figure 6. High diversity of intracellular CD63 protein forms results from extended N-glycosylation*. Total protein lysates from depicted cells were untreated (-) or treated (+) with PNG-ase F, separated by SDS-PAGE and analysed by immunoblot using mCD63.07 and chemiluminescence detection. The triangle on the right points to the unmodified CD63 protein present in samples treated with PNG-ase F. The arrows indicate unknown protein bands in PNG-ase F treated samples. Positions of protein marker bands are denoted on the left.*

After PNG-ase F treatment all cells except for ko-MEF and RAW264.7 exhibited in immunoblot analysis by mCD63.07 a predominant single band under 25 kDa which corresponds to the predicted size of unmodified CD63 protein. Surprisingly, the identical band of 25 kDa was also detected in DC2.4 cells although the same amount of CD63 protein was not detected in DC2.4 sample without PNG-ase F. Even stronger effect of obtaining enhanced signal after PNG-ase F treatment was also visible in BV2 cells.

This further indicates that mCD63.07 antibody can be used to detect even low levels of CD63 protein naturally present as multiple glycoforms in cell if a deglycosylation is applied resulting in a single and more abundant CD63 band in immunoblot. The accumulation effect of deglycosylated CD63 forms was not detected in RAW264.7 under current experimental setting.

Interestingly, a faint signal below 40 kDa and a weak signal around 15 kDa were detected in PNG-ase F treated wt-MEF, SVEC4-10, P815 and B12 cell lysates. Further studies are required to determine if these bands might be additional posttranslational modifications, different splicing variant or incomplete digestion by PNG-ase F.

4.4. mCD63.07 and mCD63.08 can specifically detect CD63 protein *in situ* in primary mouse embryonic fibroblasts

Previous results (Figures 4-7) have shown that the newly produced mCD63.07 antibody can recognize many glycoforms of the CD63 protein if proteins are analysed in detergent-solubilized, denatured and reduced conditions like in western blot analysis. Such characterisation of CD63 glycoforms is further confirmed by another newly produced mouse monoclonal antibody, mCD63.08, which can also specifically recognise various CD63 glycoforms in western blot analysis and provide comparable results to mCD63.07 (data not shown). Therefore, existence of multiple CD63-glycoforms within the cell prompts a question of potential novel CD63 cellular localisation and functions. Of note, the current literature associates CD63 with many cellular functions, yet CD63 is regarded as a resident protein of late endosomes and lysosomes.

The method of choice for localising proteins in cells is immunofluorescence. However, the standard immunofluorescence protocol including cell fixation, permeabilisation and antibody incubation did not

provide any signals with either of the newly produced anti-CD63 antibodies (data not shown). To enable CD63 protein detection by mCD63.07 and mCD63.08 in intact cells, an in-house protocol for immunofluorescence analysis was specially developed after extensive optimisation starting with published protocol (33). The crucial new steps in optimised protocol were to treat cells first with urea antigen retrieval (AR) and then with PNG-ase F.

To test the antibody specificity as well as to ensure that harsh cell treatments did not result in a false positive signal, the immunofluorescence signal of mCD63.07 and mCD63.08 was first compared between wt-MEF and ko-MEF (Figure 8).

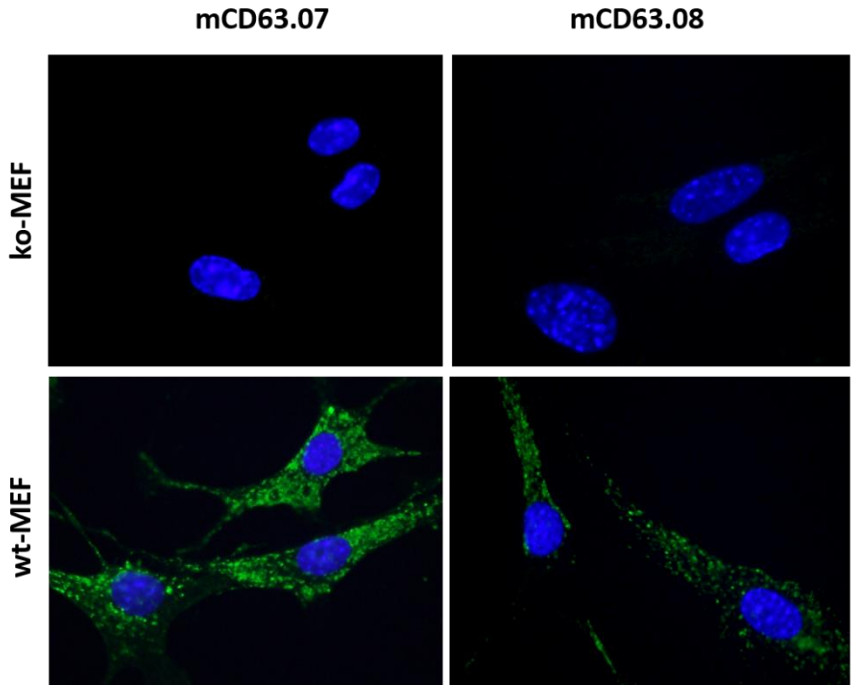

Figure 7. Knockout validation of mCD63.07 and mCD63.08 for usage in immunofluorescence. *Primary wild type (wt) and CD63-knockout (ko) mouse embryonic fibroblasts (MEF) were seeded on cover slips, fixed with PFA, treated with urea antigen retrieval and permeabilised with Triton-X100. After PNG-ase F treatment, cells were blocked with 5% BSA. CD63 was visualised using purified mCD63.07 or mCD63.08 and Alexa Fluor 488 goat anti-mouse IgG secondary antibody. Cell nuclei were visualised with DAPI. Images were analysed by fluorescence microscopy (Olympus BX51, magnification 1000x). The images shown above are representative of two experiments.*

Both mCD63.07 and mCD63.08 gave a positive signal on wt-MEF, while no signal could be detected on ko-MEF. The signal detected by both antibodies in wt-MEF consisted of small and dense granule-like structures evenly spread throughout the cell, including regions next to plasma membrane and in extended filopodia. Due to the irregular and disperse appearance of the CD63 signal in this experimental setting, it is not possible to define the cell location of the CD63 protein. Although mCD63.08 appears to produce a weaker signal than mCD63.07, further titration of antibody concentration is required to achieve optimal results for both antibodies.

4.5. High sensitivity of mCD63.07 and mCD63.08 antibodies after antigen retrieval and PNG-ase F treatment on cells *in vitro*

The previous immunofluorescence analysis (Figure 8) has successfully validated the specificity of mCD63.07 and mCD63.08 on wt-MEF and ko-MEF. To prove the effectiveness of the novel AR-based immunofluorescence protocol on other cells and to learn more about cellular localisation of CD63, the next immunofluorescence experiments were performed on immortalised murine fibroblast (B12) cell line (Figure 9). Both mCD63.07 and mCD63.08 were applied in serial dilutions from 1:100 to 1:800. CD63 signal was detected in B12 cells in a form of brightly stained granules located mainly around the cell nucleus and to a lesser extent on the cell periphery. Plasma membrane staining was not detected.

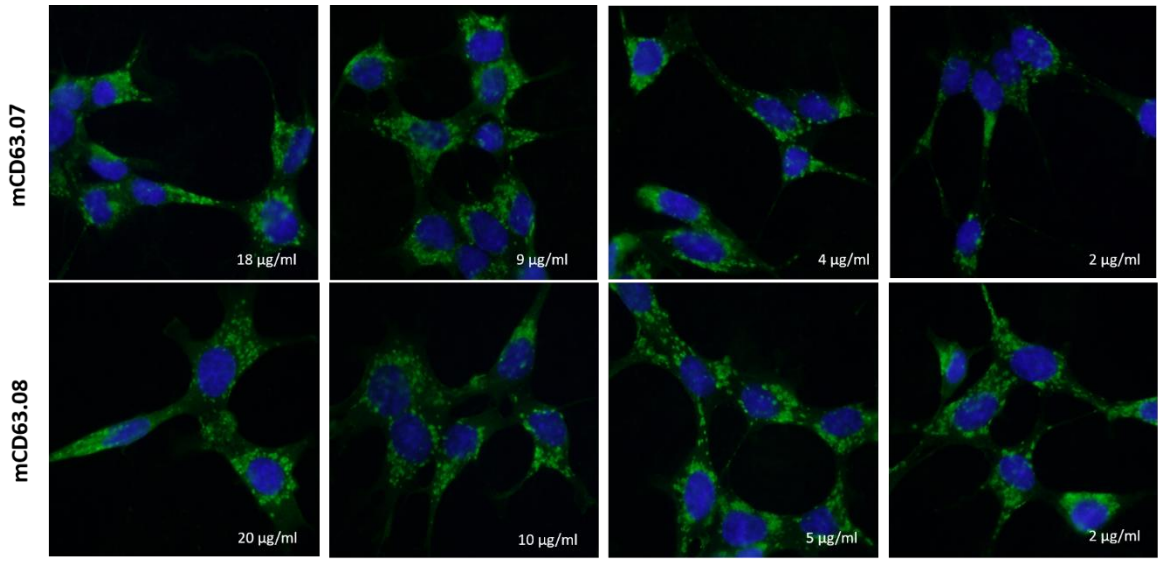

Figure 8. mCD63.07 and mCD63.08 titration. *B12 cells were fixed, treated with urea antigen retrieval and permeabilised. After permeabilisation, proteins were deglycosylated with PNG-ase F and blocked with 5% BSA. CD63 was visualised using mCD63.07 or mCD63.08 (concentrations denoted in the bottom left corner for each dilution) and Alexa Fluor 488 goat anti-mouse IgG secondary antibody (1:1000, Invitrogen). Cell nuclei were visualised with DAPI. Images were analysed by fluorescence microscopy (Olympus BX51, magnification 1000x). The images shown above are representative of three antibody titration experiments.*

Signals of both antibody titrations were quantified (Figure 10). Titration of mCD63.07 showed inclination to signal decline starting from 1:400. However, this slight decrease in corrected total cell fluorescence (CTCF) at higher dilutions was not statistically significant due to high standard deviations. When the overall CTCF values were compared for the two antibodies (Figure 10) no statistically significant difference in CTCF values was observed.

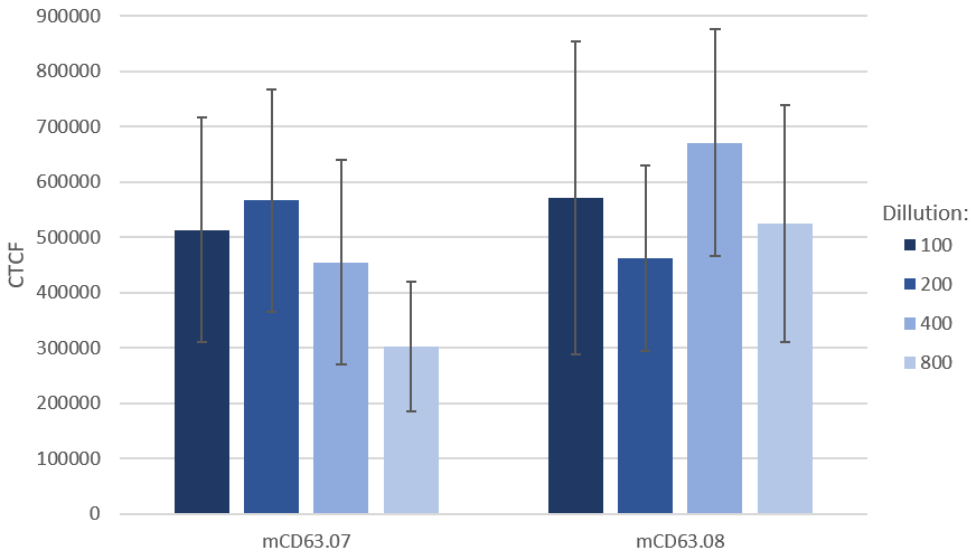

***Figure 9. Antibody titrations yield inconclusive results**. Graphical representation of the quantification of immunofluorescence analysis results for CD63. The graph shows a representative experiment of 3 experiments made. Quantification of immunofluorescence was done in the ImageJ programme. Marked on the y-axis is the CTCF calculated for 100 cells for each dilution. The bars represent standard deviation values for each dilution.*

4.6. mCD63.07 and mCD63.08 as potential tool to discover novel localisations and mechanisms of CD63 action

This study describes detection of cellular CD63 *in situ* by using newly developed antibodies and specialised protocol based on denaturation and deglycosylation of proteins. With such original approach CD63 might be visualised in cellular parts other than late endosomes and lysosomes which are traditionally considered as main organelles for CD63 localisation. Thus, to gain deeper insight into CD63 localisation, visualisation of cellular CD63 was continued by confocal microscopy where B12 cells treated with AR-based protocol and PNG-ase F were imaged after staining with mCD63.07 antibody (Figure 11).

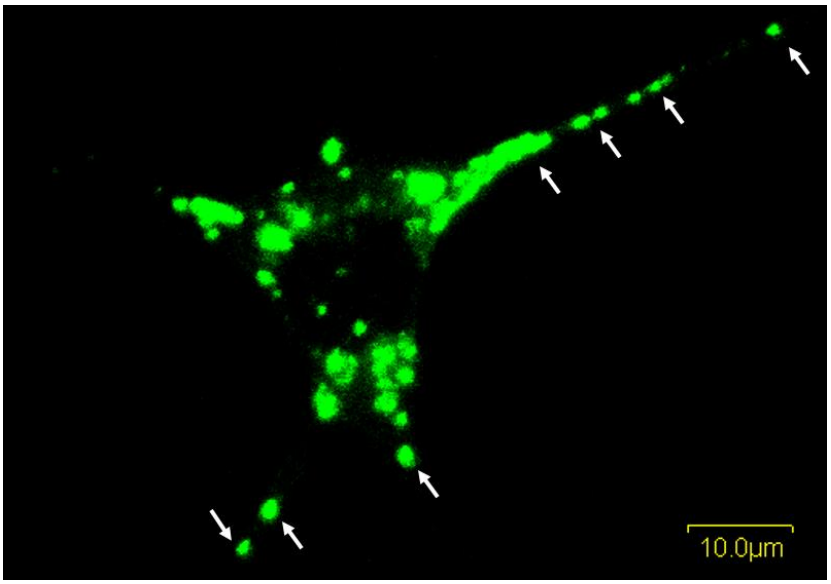

Figure 10. CD63 detected by mCD63.07 in cell filopodia. *Coverslips with seeded B12 cells were treated with antigen retrieval and PNG-ase F, followed by staining with mCD63.07 and anti-mouse Alexa 488. Images were taken by confocal microscopy (Olympus FV3000). The 3D overlap of 9 slices from confocal microscope images was produced in the ImageJ programme. The displayed cell is a representative sample of cells analysed by confocal microscopy. The arrows point to CD63 in cell filopodia. Scale bar: 10 µm.*

CD63 in B12 cells was detected in the form of large granules and as a dispersed signal within the cell body while the arrows in Figure 11 point to small granules present in cell filopodia. Granules also often appeared in the filopodia that cells extended towards each other (data not shown). No signal for CD63 was detected on the plasma membrane. To better evaluate if mCD63.07 and mCD63.08 have potential to discover CD63 in new cellular localisation, CD63 was further detected in B12 cells by commonly used commercial anti-CD63 antibody (Figure 12). The commercial antibody was applied in 4 different protocols: 1) standard; 2) PNG-ase F; 3) antigen retrieval (AR); 4) AR + PNG-ase F.

Both standard and PNG-ase F protocol resulted in a similar signal consisting of granular cluster located next to the nucleus in most cells. Less dense granular groups or sporadic dots were present further from nucleus,

but no signal in filapodia was detected. Protocols including antigen retrieval did not result in any signal. These initial comparisons indicate that neither applied commercial antibody nor newly developed antibodies bind to sugar moiety, but to protein part of CD63 glycoprotein.

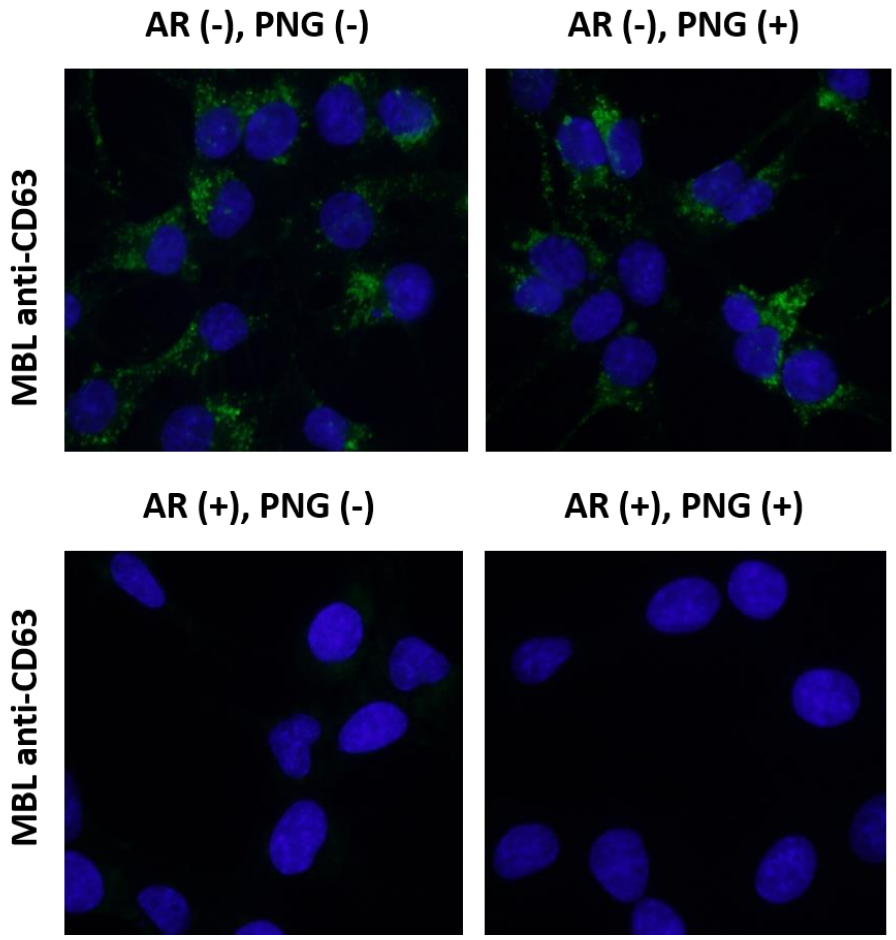

Figure 11. Commercial anti-CD63 mAb binds to a nonlinear epitope on protein moiety of the CD63 glycoprotein. *B12 cells were seeded on cover slips, fixed with PFA and separately treated by indicated protocols which could include antigen retrieval (AR) and treatment with PNG-ase F (PNG). All slides were permeabilised with TritonX-100, blocked with 5% BSA and incubated with rat anti-CD63 (MBL) and Alexa Fluor 488 goat anti-rat IgG secondary antibody (Invitrogen). Images were analysed by fluorescence microscopy (Olympus BX51, magnification 1000x).*

5. DISCUSSION

CD63 is involved in a variety of cellular processes and interacts with a number of proteins (7). However, it is not known how CD63 achieves such diverse cellular functions because detailed biochemical characterisations are hampered due to lack of appropriate CD63 antibodies. Commercial anti-CD63 antibodies do not produce a reproducible signal for CD63 on western blot. Some antibodies detect one band while others detect a range of bands that varies depending on the manufacturer (34), (35), (36). Thus, two new murine monoclonal antibodies directed against the EC2 domain of mouse CD63 protein were developed at the Center for Proteomics (University of Rijeka). Briefly, the antibodies (mCD63.07 and mCD63.08) were produced by immunizing mice with recombinant CD63 synthesized in human embryonic kidney 293 cells and purified by affinity chromatography.

The specificity of the purified mouse monoclonal mCD63.07 antibody for CD63 protein was examined by western blot analysis. Whole cell protein extracts were isolated from wild type and CD63 knockout MEFs, analysed by western blot and immunodetected with anti-actin antibody to verify that similar protein amounts were loaded for both samples (Figure 4). The membrane was immunodetected with mCD63.07. and even at the exposition of 10 minutes with the strongest reagent, mCD63.07 detected multiple proteins of different sizes in wild type cells, but none in CD63 knockout cells (Figure 4), proving thereby to be highly specific. The specificity of the purified mouse monoclonal mCD63.08 antibody for CD63 protein was also verified by western blot analysis (data not shown). After the antibody was shown to be specific, systematic comparison of CD63 expression among different cell lines was performed with mCD63.07. Protein extracts from six murine cell lines of diverse tissue origin were separeted by SDS-PAGE and immunodetected with mCD63.07 (Figure 5). Detected proteins differed among cells not only in size, ranging roughly

from 35 kDa to more than 100 kDa, but also in detected levels for signals throughout the size range.

To get a more detailed overview of CD63 protein forms, CD63-positive cell lines were analysed by western blot where all protein bands under 35 kDa were released from the gel during electrophoresis (Figure 6). The markings in Figure 6 point to CD63 protein bands that appear only in some cell lines at certain molecular weights which implies that each cell type could have specific forms of CD63 protein. In this context, DC2.4 and wt-MEF cell lines are interesting because they contain protein bands (marked with a triangle and an arrow) that are not visible elsewhere, and it is possible that these bands represents protein forms of CD63 specific for DC2.4 and wt-MEF cells.

Due to CD63 being a heavily glycosylated protein, the detected protein bands in Figures 5 and 6 were assumed to be various CD63 glycoforms. To confirm this assumption, a glycosylation analysis was performed where protein extracts were treated with PNG-ase F prior to immunodetection (Figure 7). After PNG-ase F enzymatically cleaved N-linked glycans, the detected CD63 signal (marked with a triangle) in all cells was at roughly 25 kDa, corresponding to the predicted molecular weight of unmodified CD63 protein. PNG-ase F treatment confirmed that N-glycosylation represents the majority of CD63 posttranslational modifications and that the various protein forms detected in untreated protein extracts represent CD63 glycoforms. The arrows in Figure 7 point to protein bands in PNG-ase F-treated samples that may indicate the presence of some other CD63 posttranslational modification, splicing variant (37) or incomplete PNG-ase F enzymatic reaction. Perhaps some of the glycoforms visible on western blots represent immature CD63 forms released from the endoplasmatic reticulum during cell lysis (38). The shortcoming of western blot is that protein extraction depends on the type of detergent used, especially for transmembrane proteins such as CD63.

The extraction of CD63 depends on the composition of the membrane around it. If the lipid content around CD63 is not soluble in the detergent of our choice, then that protein form will not be extracted from the cell lysate. Therefore, it is very likely that not all forms of CD63 have been isolated by the standard protein extraction procedure described in materials and methods. This could be improved by optimising the extraction process by testing different conditions and detergents.

Since the new antibodies provide a similar signal in western blot analysis, their usability was tested in the immunofluorescence method to check whether they provide a different information about the location of CD63 in the cell. It was found that novel antibodies provide a positive signal in immunofluorescence, but only when the CD63 antigen is in a linear conformation obtained by denaturation and reduction and there were no oligosaccharide residues to obstruct the antibodies from reaching their epitope on fixed cells. To achieve such conditions for CD63, a new immunofluorescence protocol was developed where cellular proteins were denatured, reduced, and deglycolised before immunodetection. To verify antibody specificity, wild type and CD63 knockout MEFs were cultivated, fixed, denatured and reduced with urea antigen retrieval, permeabilised, deglycosylated and immunodetected with mCD63.07 and mCD63.08 (Figure 8). For both antibodies, a positive signal was present in wild type MEFs and absent in knockout MEFs which proved that novel antibodies specifically detect CD63 in immunofluorescence. In addition to the fact that the new antibodies were shown to be specific, they proved that the various treatments to which the cells were subjected did not lead to false positive signals. The scattered CD63 signal detected in wt-MEFs (Figure 8) was not characteristic of CD63 and it was assumed that the denaturation treatment was too harsh for wt-MEFs. Due to the fact that wt-MEFs are a primary cell line, the cultivation of which can be unpredictable, the following immunofluorescence experiments were performed on more resistant B12 cells.

To find out the optimal antibody concentration of mCD63.07 and mCD63.08 for immunofluorescence analysis of CD63, three antibody titration experiments were performed on B12 cells (Figure 9). According to the images from the fluorescent microscope, the antibodies seemed to provide the same signal in all dilutions and therefore the signal was quantified in the ImageJ programme (Figure 10). Quantification of the immunofluorescent signal confirmed that antibody dilutions did not lead to a decrease in the signal intensity for CD63. It was later discovered in literature that the antigen retrieval method allows the use of 5-10 times lower antibody concentrations than is usual for immunofluorescence (33). Antigen linearisation, especially in combination with deglycosylation, enables better access of the antibody to the target epitope which allows the application of much lower antibody concentrations. Based on the titration results so far, the antibodies can be successfully used even at the concentration of 2 µg/ml.

To better assess the location of the CD63 protein in B12 cells, and to see if the new antibodies detect another form of CD63 than is traditionally described in literature (7), coverslips where proteins were denatured and deglycosylised were analysed by confocal microscopy (Figure 11). mCD63.07 detects CD63 in the form of large granules located within the cell body, and small granules in the cell filopodia. Also, a disperse signal was detected in the cell body, while there was no signal for CD63 at the plasma membrane. mCD63.08 provides a similar signal for CD63 when analysed by confocal microscopy (data not shown). The last immunofluorescence experiment was performed on B12 cells where a commercial anti-CD63 antibody was used for immunodetection (Figure 12) in four different protocols. This experiment verified that antigen retrieval dentures proteins because the commercial antibody was not effective on cells that have been treated with antigen retrieval, suggesting that this antibody binds to CD63 only when its target epitope is in a nonlinear conformation. The commercial antibody detected a granular from of CD63

located around the cell nucleus, but no CD63-positive granules were detected in cell filopodia.

Immunofluorescence allows the localisation CD63 protein, but not the study of specific protein forms. Immunofluorescence has a similar drawback as western blot. During cell permeabilisation, membrane parts are dissolved and washed away, particularly affecting CD63 because it is a transmembrane protein. Furthermore, this complicates the detection of CD63 on the plasma membrane. This shortcoming could be resolved by finding a less harsh detergent for permeabilisation or avoiding permeabilisation altogether.

The novel antibodies, mCD63.07 and mCD63.08, are highly specific and sensitive in western blot and immunofluorescence and represent useful tools for further characterisation of CD63. Furthermore, these results provide a systematic comparison of CD63 proteins among different proliferative cells and indicate that CD63 glycosylation is cell-type specific. In the context of EV research, cell-type specific CD63 glycosylation offers a new momentum to the development of noninvasive diagnostic procedures based on EVs positive for CD63. The production of antibodies for cell-type specific CD63 glycoforms might enable isolation and further analysis of extracellular vesicles from a particular tissue (39), (40). Moreover, in proteins that are highly glycosylated, the change of their glycosylation status may provide insight into a particular condition or developmental status (41), (42), (43). Since mCD63.07 and mCD63.08 antibodies proved to be useful tools in the study of CD63, it would be interesting to use them to determine whether the expression of CD63 glycoforms changes under the influence of cellular stress caused by inflammation or infection.

6. CONCLUSION

This thesis characterised two newly produced mouse monoclonal antibodies, mCD63.07 and mCD63.08, targeted against mouse CD63 protein with the following findings:

1. mCD63.07 antibody specifically detects both glycosylated and unglycosylated forms of mouse CD63 protein in immunoblot analysis after SDS-PAGE, indicating that the epitope is a linear peptide sequence;

2. As detected by mCD63.07 antibody, CD63 is present in cell not as a single form of protein but as a collection of multiple glycosylated forms which can differ in quantity and size ranging from 30 kDa to more then 100 kDa depending on cell type and with a potential that some of these CD63 glycoforms are cell-type specific;

3. mCD63.07 and mCD63.08 can specifically localise CD63 in cells by immunofluorescence analyses after cells have been treated by antigen retrieval and deglycosylation;

4. The immunofluorescence detection of CD63 by novel antibodies indicates a far broader localisation of CD63 in cell then in late endosomes and lysosomes as most commonly described in literature.

Taken together, these results suggest that the newly developed and knock-out validated antibodies mCD63.07 and mCD63.08 possess necessary specificity and sensitivity required for future systematic biochemical characterisation of CD63.

7. REFERENCES

1. Charrin S, Jouannet S, Boucheix C, Rubinstein E. Tetraspanins at a glance. J Cell Sci. 2014 Sep 1;127(Pt 17):3641–8.

2. Hemler ME. Tetraspanin functions and associated microdomains. Nat Rev Mol Cell Biol. 2005 Oct;6(10):801–11.

3. Andreu Z, Yáñez-Mó M. Tetraspanins in extracellular vesicle formation and function. Front Immunol. 2014;5:442.

4. Stipp CS, Kolesnikova TV, Hemler ME. Functional domains in tetraspanin proteins. Trends Biochem Sci. 2003 Feb;28(2):106–12.

5. Zimmerman B, Kelly B, McMillan BJ, Seegar TCM, Dror RO, Kruse AC, et al. Crystal Structure of a Full-Length Human Tetraspanin Reveals a Cholesterol-Binding Pocket. Cell. 2016 03;167(4):1041-1051.e11.

6. Termini CM, Gillette JM. Tetraspanins Function as Regulators of Cellular Signaling. Front Cell Dev Biol [Internet]. 2017 Apr 6 [cited 2020 May 5];5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5382171/

7. Pols MS, Klumperman J. Trafficking and function of the tetraspanin CD63. Exp Cell Res. 2009 May 15;315(9):1584–92.

8. Liang Y, Eng WS, Colquhoun DR, Dinglasan RR, Graham DR, Mahal LK. Complex N-linked glycans serve as a determinant for exosome/microvesicle cargo recruitment. J Biol Chem. 2014 Nov 21;289(47):32526–37.

9. Schröder J, Lüllmann-Rauch R, Himmerkus N, Pleines I, Nieswandt B, Orinska Z, et al. Deficiency of the tetraspanin CD63 associated with kidney pathology but normal lysosomal function. Mol Cell Biol. 2009 Feb;29(4):1083–94.

10. Hildreth JE, Derr D, Azorsa DO. Characterization of a novel self-associating Mr 40,000 platelet glycoprotein. Blood. 1991 Jan 1;77(1):121–32.

11. Israels SJ, McMillan-Ward EM. Palmitoylation supports the association of tetraspanin CD63 with CD9 and integrin alphaIIbbeta3 in activated platelets. Thromb Res. 2010 Feb;125(2):152–8.

12. Cooper GM. The Cell. 2nd ed. Sinauer Associates; 2000.

13. Rous BA, Reaves BJ, Ihrke G, Briggs JAG, Gray SR, Stephens DJ, et al. Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. Mol Biol Cell. 2002 Mar;13(3):1071–82.

14. Knol EF, Mul FP, Jansen H, Calafat J, Roos D. Monitoring human basophil activation via CD63 monoclonal antibody 435. J Allergy Clin Immunol. 1991 Sep;88(3 Pt 1):328–38.

15. Duffield A, Kamsteeg E-J, Brown AN, Pagel P, Caplan MJ. The tetraspanin CD63 enhances the internalization of the H,K-ATPase beta-subunit. Proc Natl Acad Sci U S A. 2003 Dec 23;100(26):15560–5.

16. Takino T, Miyamori H, Kawaguchi N, Uekita T, Seiki M, Sato H. Tetraspanin CD63 promotes targeting and lysosomal proteolysis of membrane-type 1 matrix metalloproteinase. Biochem Biophys Res Commun. 2003 Apr 25;304(1):160–6.

17. Flannery AR, Czibener C, Andrews NW. Palmitoylation-dependent association with CD63 targets the Ca2+ sensor synaptotagmin VII to lysosomes. J Cell Biol. 2010 Nov 1;191(3):599–613.

18. Yoshida N, Kitayama D, Arima M, Sakamoto A, Inamine A, Watanabe-Takano H, et al. CXCR4 expression on activated B cells is downregulated by CD63 and IL-21. J Immunol. 2011 Mar 1;186(5):2800–8.

19. Kraft S, Jouvin M-H, Kulkarni N, Kissing S, Morgan ES, Dvorak AM, et al. The tetraspanin CD63 is required for efficient IgE-mediated mast cell degranulation and anaphylaxis. J Immunol. 2013 Sep 15;191(6):2871–8.

20. Doyle EL, Ridger V, Ferraro F, Turmaine M, Saftig P, Cutler DF. CD63 is an essential cofactor to leukocyte recruitment by endothelial P-selectin. Blood. 2011 Oct 13;118(15):4265–73.

21. Tugues S, Honjo S, König C, Padhan N, Kroon J, Gualandi L, et al. Tetraspanin CD63 promotes vascular endothelial growth factor receptor 2-β1 integrin complex formation, thereby regulating activation and downstream signaling in endothelial cells in vitro and in vivo. J Biol Chem. 2013 Jun 28;288(26):19060–71.

22. Lee SY, Kim JM, Cho SY, Kim HS, Shin HS, Jeon JY, et al. TIMP-1 modulates chemotaxis of human neural stem cells through CD63 and integrin signalling. Biochem J. 2014 May 1;459(3):565–76.

23. Hotta H, Hara I, Miyamoto H, Homma M. Overexpression of the human melanoma-associated antigen ME491 partially suppresses in

vivo malignant phenotypes of H-ras-transformed NIH3T3 cells in athymic nude mice. Melanoma Res. 1991 Jul;1(2):125–32.

24. Rorive S, Lopez XM, Maris C, Trepant A-L, Sauvage S, Sadeghi N, et al. TIMP-4 and CD63: new prognostic biomarkers in human astrocytomas. Mod Pathol. 2010 Oct;23(10):1418–28.

25. Kwon MS, Shin S-H, Yim S-H, Lee KY, Kang H-M, Kim T-M, et al. CD63 as a biomarker for predicting the clinical outcomes in adenocarcinoma of lung. Lung Cancer. 2007 Jul;57(1):46–53.

26. Lai X, Gu Q, Zhou X, Feng W, Lin X, He Y, et al. Decreased expression of CD63 tetraspanin protein predicts elevated malignant potential in human esophageal cancer. Oncol Lett. 2017 Jun;13(6):4245–51.

27. Xia Z, Zhang S, Zhou Z. Relation of ME491/CD63 gene and integrin alpha5 in the invasion and metastases of ovarian cancer. Zhonghua Fu Chan Ke Za Zhi. 2005 Nov;40(11):765–9.

28. Loss of CD63 expression as a prognostic marker in breast cancer [Internet]. ResearchGate. [cited 2020 Jul 11]. Available from: https://www.researchgate.net/publication/296400117_Loss_of_CD63_expression_as_a_prognostic_marker_in_breast_cancer

29. Koh H, An H, Jung J, Song D. The prognostic significance of CD63 expressionin patients with non-small cell lung cancer. Pol J Pathol. 2019;70(3):183–8.

30. van Niel G, Charrin S, Simoes S, Romao M, Rochin L, Saftig P, et al. The tetraspanin CD63 regulates ESCRT-independent and -dependent endosomal sorting during melanogenesis. Dev Cell. 2011 Oct 18;21(4):708–21.

31. Källquist L, Hansson M, Persson A-M, Janssen H, Calafat J, Tapper H, et al. The tetraspanin CD63 is involved in granule targeting of neutrophil elastase. Blood. 2008 Oct 15;112(8):3444–54.

32. Nishibori M, Cham B, McNicol A, Shalev A, Jain N, Gerrard JM. The protein CD63 is in platelet dense granules, is deficient in a patient with Hermansky-Pudlak syndrome, and appears identical to granulophysin. J Clin Invest. 1993 Apr;91(4):1775–82.

33. Hayashi T, Lewis A, Hayashi E, Betenbaugh MJ, Su T-P. Antigen retrieval to improve the immunocytochemistry detection of sigma-1 receptors and ER chaperones. Histochem Cell Biol. 2011 Jun;135(6):627–37.

34. Takawale A, Zhang P, Patel VB, Wang X, Oudit G, Kassiri Z. Tissue Inhibitor of Matrix Metalloproteinase-1 Promotes Myocardial Fibrosis by Mediating CD63-Integrin β1 Interaction. Hypertension. 2017 Jun;69(6):1092–103.

35. Lupia A, Peppicelli S, Witort E, Bianchini F, Carloni V, Pimpinelli N, et al. CD63 tetraspanin is a negative driver of epithelial-to-mesenchymal transition in human melanoma cells. J Invest Dermatol. 2014 Dec;134(12):2947–56.

36. Kudo K, Yoneda A, Sakiyama D, Kojima K, Miyaji T, Yamazaki M, et al. Cell surface CD63 increased by up-regulated polylactosamine modification sensitizes human melanoma cells to the BRAF inhibitor PLX4032. FASEB J. 2019 Mar;33(3):3851–69.

37. Hochheimer N, Sies R, Aschenbrenner AC, Schneider D, Lang T. Classes of non-conventional tetraspanins defined by alternative splicing. Scientific Reports. 2019 Oct 1;9(1):14075.

38. Määttänen P, Gehring K, Bergeron JJM, Thomas DY. Protein quality control in the ER: the recognition of misfolded proteins. Semin Cell Dev Biol. 2010 Jul;21(5):500–11.

39. Schäfer T, Starkl P, Allard C, Wolf RM, Schweighoffer T. A granular variant of CD63 is a regulator of repeated human mast cell degranulation. Allergy. 2010 Oct;65(10):1242–55.

40. Haji-Ghassemi O, Blackler RJ, Martin Young N, Evans SV. Antibody recognition of carbohydrate epitopes†. Glycobiology. 2015 Sep;25(9):920–52.

41. Hollander N, Haimovich J. Altered N-Linked Glycosylation in Follicular Lymphoma and Chronic Lymphocytic Leukemia: Involvement in Pathogenesis and Potential Therapeutic Targeting. Front Immunol. 2017;8:912.

42. Tuccillo FM, de Laurentiis A, Palmieri C, Fiume G, Bonelli P, Borrelli A, et al. Aberrant glycosylation as biomarker for cancer: focus on CD43. Biomed Res Int. 2014;2014:742831.

43. Engering A, Kuhn L, Fluitsma D, Hoefsmit E, Pieters J. Differential post-translational modification of CD63 molecules during maturation of human dendritic cells. Eur J Biochem. 2003 Jun;270(11):2412–20.

8. CURRICULUM VITAE

PERSONAL INFORMATION

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EDUCATION AND TRAINING

2018–Present — M.Sc., Master Studies, Biotechnology in Medicine
Department of Biotechnology at University of Rijeka, Rijeka (Croatia)

2015–2018 — B.Sc., Undergraduate Studies, Biology and Chemistry
Faculty of Science at University of Split, Split (Croatia)

2011–2015 — Highschool
IV. Gymnasium Marko Marulić, Split (Croatia)

PERSONAL SKILLS

Mother tongue(s): Croatian

Foreign language(s)

	UNDERSTANDING		SPEAKING		WRITING
	Listening	Reading	Spoken interaction	Spoken production	
English	C2	C1	C1	B2	B2
German	A2	A2	A1	A1	A1

Levels: A1 and A2: Basic user - B1 and B2: Independent user - C1 and C2: Proficient user
Common European Framework of Reference for Languages - Self-assessment grid

Digital skills

SELF-ASSESSMENT				
Information processing	Communication	Content creation	Safety	Problem-solving
Independent user	Independent user	Basic user	Independent user	Independent user

Digital skills - Self-assessment grid

Independent user of: MS Office.

Basic user of: ImageJ.

Driving licence B

ADDITIONAL INFORMATION

Training

August 2019 – June 2020, Rijeka, Croatia

Department of Physiology, Immunology and Patophysiology, Faculty of Medicine, University of Rijeka

As a part of Master's thesis related work I performed the following methods: western blot, immunofluorescence and cell fractionation.

Congresses

24-26 April, 2020, Rijeka, Croatia

Student Congress of Neuroscience – NEURI

The abstract was accepted, but due to the COVID-19 outbreak the event was canceled

21-23 May, 2020, Graz, Austria

International Student Congress'- ISC

The abstract was accepted, but due to the COVID-19 outbreak the event was canceled

Volunteering

2019, Rijeka, Croatia

Festival of Science at the Department of Biotechnology at University of Rijeka

2018, Split, Croatia

Festival of Science at the Faculty of Science at University of Split

Memberships

2018-Present

Member of the Association of Biotechnology Students of the University of Rijeka

Honours and awards

2019/2020

Scholarship of the City of Kaštela for success

2019

Dean's Award for Success in Undergraduate Studies in Biology and Chemistry, Faculty of Science at University of Split, Split (Croatia)

2018/2019

Scholarship of the City of Kaštela for success

2017/2018

Ministry of Science and Education of the Republic of Croatia - STEM Scholarship

2015/2016

Scholarship of the City of Kaštela for success