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Co-Mentor: Assist. Prof. Ilija Brizić

SVEUČILIŠTE U RIJECI
ODJEL ZA BIOTEHNOLOGIJU
Diplomski sveučilišni studij
Biotehnologija u medicini

Vedrana Krušić

Novoproizvedeno mišje monoklonsko protutijelo prepoznaje tkivno specifične glikoforme markera izvanstaničnih vezikula CD63 u mišjim tkivima

Diplomski rad

Rijeka, 2020.

Mentor: izv. prof. dr. sc. Kristina Grabušić
Komentor: doc. dr. sc. Ilija Brizić

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Mentor: Assoc. Prof. Kristina Grabušić

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Master's thesis was defended on the 19th June, 2020, in front of the committee:

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Summary

CD63 is a ubiquitously expressed transmembrane glycoprotein frequently used as a general protein marker of extracellular vesicles (EVs). EVs are cell-secreted and membrane-enclosed nanoparticles which circulate through the blood and other body fluids and thus are available for isolation. EV cargo is very dynamic and reflects the biological status of the originating tissue, which, coupled with their accessibility from body fluids, could make EVs a valuable diagnostic and prognostic tool. However, identification of tissue-specific EVs is still lacking.

The goal of this study was to examine the tissue specificity of CD63 protein using a novel murine monoclonal antibody mCD63.07 produced at the Center for Proteomics, University of Rijeka, Faculty of Medicine, Croatia. Western blot analysis of salivary gland, brain, lung, liver, kidney, muscle, bone marrow and spleen tissue obtained from wild type (wt) mice revealed that the expression levels and protein forms of CD63 varied among murine tissues. Molecular weight of detected CD63 forms ranged from 25 to 170 kDa, depending on the tissue, whereby some of CD63 forms were tissue-specific. A comparison with corresponding knockout tissues confirmed the high specificity of mCD63.07 antibody. PNG-ase F deglycosylation treatment of proteins from salivary gland, kidney, bone marrow, liver, muscle and spleen wt samples resulted in merging of higher molecular weight protein bands into one band at approximately 24 kDa. Thus the observed differences in detected CD63 forms arise from tissue-specific N-glycosylation of the CD63 protein.

The findings indicate that CD63 glycoforms are tissue-specific and therefore might be used for isolation of tissue-specific EVs. Future characterization of human tissue-specific glycoforms of CD63 could enable the development of new EV-related blood-based diagnostic methods.

Key words: CD63, tetraspanins, glycoproteins, extracellular vesicles

Sažetak

CD63 je ubikvitarno eksprimirani transmembranski glikoprotein, najčešće korišten kao opći proteinski marker izvanstaničnih vezikula (IV). IV-e su membranom obavijene nanočestice koje stanice izlučuju u krv i druge tjelesne tekućine, što ih čini lako dostupnima za izolaciju. Sadržaj IV-a je vrlo dinamičan i odražava biološki status tkiva iz kojih potječu, što u kombinaciji s njihovom lako dostupnošću može učiniti IV-e vrijednim dijagnostičkim i prognostičkim alatom. Međutim, identifikacija tkivno-specifičnih IV-a još uvijek nije moguća.

Cilj ove studije bio je ispitati tkivnu specifičnost proteina CD63 pomoću novog mišjeg monoklonskog antitijela mCD63.07 proizvedenog u Centru za proteomiku Medicinskog fakulteta u Rijeci. Western blot analiza tkiva žlijezde slinovnice, mozga, pluća, jetre, bubrega, mišića, koštane srži i slezene izoliranih iz miševa divljeg tipa otkrila je da se razina ekspresije i proteinski oblici CD63 razlikuju u mišjim tkivima. Molekularna masa detektiranih oblika CD63 kretala se u rasponu od 25 do 170 kDa, ovisno o tkivu, te su neki od oblika pokazali tkivno-specifičnu ekspresiju. Usporedba s odgovarajućim *knockout* tkivima potvrdila je visoku specifičnost antitijela mCD63.07. Deglikozilacijski tretman proteina iz žlijezde slinovnice, bubrega, koštane srži, jetre, mišića i slezine rezultirao je spajanjem proteinskih *bendova* veće molekularne mase u jedan *bend* od približno 24 kDa, što potvrđuje da uočene razlike u opaženim oblicima CD63 proteina potječu iz tkivno-specifične N-glikozilacije proteina CD63.

Ovi rezultati pokazuju da su glikoforme proteina CD63 tkivno-specifične te se stoga mogu koristiti za izolaciju tkivno-specifičnih IV-a. Buduća karakterizacija glikoformi CD63 specifičnih za ljudska tkiva mogla bi omogućiti razvoj novih dijagnostičkih metoda temeljenih na izolaciji IV-a iz krvi.

Ključne riječi: CD63, tetraspanini, glikoproteini, izvanstanične vezikule

Contents

1. Introduction

1.1. The tetraspanin family of proteins

1.1.1. Structural features of the tetraspanin family

Tetraspanins, also known as transmembrane 4 superfamily proteins, are small membrane-spanning proteins. The tetraspanin superfamily consists of 33 members identified in the human proteome and is characterized by the conserved structure of four transmembrane (TM) domains, two extracellular (EC) loops of unequal size, a small intracellular loop and short intracellular tails, as shown in Figure 1 (1).

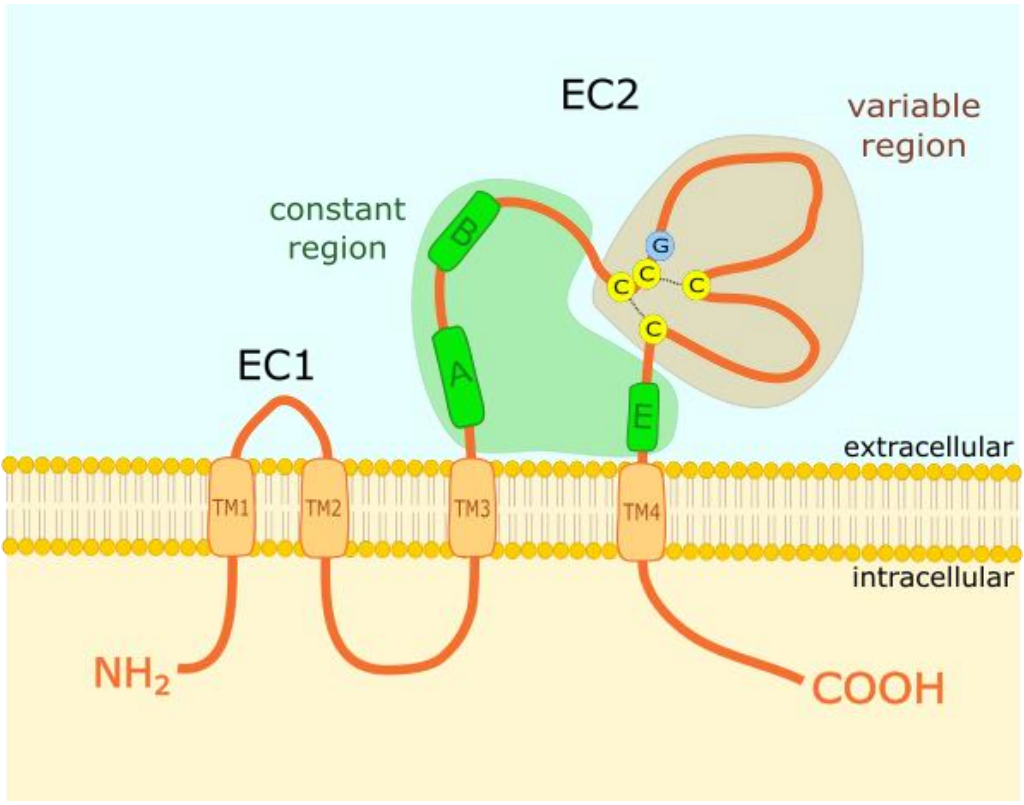


Figure 1. Structural features of tetraspanins. All tetraspanins consist of 4 transmembrane domains (TM1, TM2, TM3 and TM4), small extracellular loop (EC1), big extracellular loop (EC2), small intracellular loop and 2 short cytoplasmic tails. The EC2 domain is divided into a constant region (green depicted α-helices A, B and E) and a variable region (orange part), which contains a conserved CCG motif and at least 2 additional cysteine residues, which form disulphide bonds (dotted lines) and stabilize tetraspanin structure. The illustration is adapted according to (1).

The first extracellular loop, EC1, is relatively small and consists of 13-31 residues. It is believed that EC1 does not participate in binding and protein-protein interactions involving tetraspanins (1). The second extracellular loop, EC2, is significantly bigger and consists of 69-132 residues, divided into a constant and a variable region (2). The constant region contains 3 conserved α-helices (A, B and E), and is primarily considered to be involved in homodimerization of the protein through hydrophobic surface interactions (1). The variable region, situated between B and E helices, appears to be the most hypervariable sequence among tetraspanins and bears the majority of functionally important interaction sites involved in tetraspanin protein-protein interactions (1–3).

To be considered a tetraspanin, a protein also has to possess several other structural features, including an conserved Cys-Cys-Gly (CCG) motif, as well as 2-6 additional cysteine residues in the EC2 domain, which form disulphide bonds and participate in the proper folding of the protein. The Pro-Xaa-Xaa-Cys (PXXC) motif, where Xaa can stand for any amino acid, can also be found in the EC2 domain of the vast majority of tetraspanin proteins (1,2). Furthermore, tetraspanins characteristically carry several highly polar residues within TM domains of the protein, which are believed to be involved in the stabilization of the tertiary structure of TM domains of the protein (1).

1.1.2. Post-translational modifications of tetraspanins

Most members of the tetraspanin family acquire multiple post-translational modifications during their biosynthetic pathway. One of the most common is N-glycosylation of the asparagine residues, usually on the EC2 domain of the protein (4). N-glycosylation is a highly conserved process, crucial for protein folding, stability and function. Although it is frequently referred to as post-translational modification, N-glycosylation is, in fact, a co-translational process (5).

During protein translation and translocation in the ER lumen, a precursor oligosaccharide is transferred onto asparagine residues of a nascent polypeptide. The oligosaccharyltransferase complex, localized at the membrane of ER, catalyses the *en bloc* transfer of oligosaccharide from the dolichol pyrophosphate onto the polypeptide after recognition of consensus sequence Asn-Xaa-Ser/Thr, where Xaa stands for any neutral amino acid except proline (5,6). It is worth noting that not all potential N-glycosylation sites get occupied. This glycosylation variability at specific glycosylation sites is termed macroheterogeneity and contributes to the larger structural diversity of N-glycoproteins (7).

In eukaryotes, the initial glycan precursor structure consists of 14 sugar residues ($Glc_3Man_9(GlcNAc)_2$) attached to dolichol pyrophosphate, a polyisoprenoid lipid firmly embedded in the ER membrane (6). Once precursor oligosaccharide has been covalently linked to a protein, glucosidase I and II remove two glucose residues from the N-linked oligosaccharide (5). Then, the resulting monoglucosylated form enters the calnexin-calreticulin cycle, which acts as a chaperone quality control system for glycoprotein folding. If the protein folds correctly, an additional glucose and a single mannose residue are removed, and glycoprotein is transported to Golgi apparatus (GA) for further processing (5,6).

The initial trimming of N-glycans in the ER is a highly conserved process implicated in the proper folding of the protein, though it does not introduce much heterogeneity to the structure of produced N-glycans (Figure 2A). On the contrary, the processing of N-glycoproteins in GA is much more variable and gives rise to an enormous diversity of different N-glycan structures (7). As the newly produced glycoprotein arrived from the ER progresses from cis- to trans-face of the GA, it is subjected to a series of extensive modifications. A wide spectrum of glycosidases and glycosyltransferases, present in cisternae of GA, sequentially remove or link different monosaccharides to N-glycan core, giving rise to three main

classes of N-linked glycans: high-mannose, complex and hybrid, shown in Figure 2B (5,8).

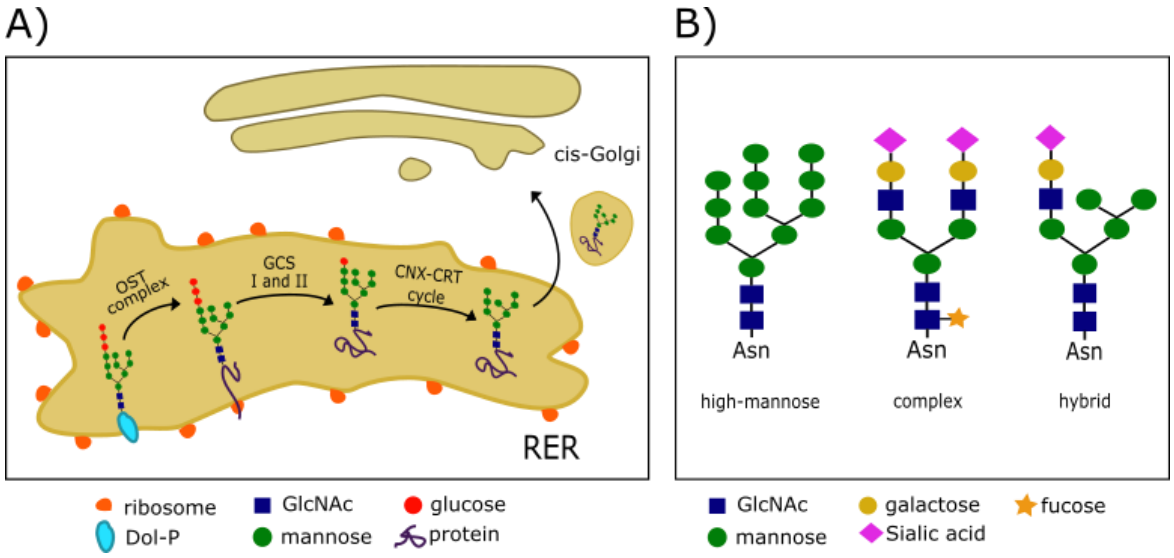


Figure 2. N-linked glycosylation in ER and main classes of N-linked glycan. (A) During protein translation and translocation in the lumen of rough endoplasmic reticulum (RER), oligosaccharyltransferase (OST) catalyses the *en bloc* transfer of precursor oligosaccharide $Glc_3Man_9(GlcNAc)_2$ from dolichol phosphate (Dol-P) to the nascent protein. Glucosidases (GCS) I and II remove two glucose residues and the protein enters calnexin-calreticulin (CNX-CRT) cycle, which results in removal of one additional glucose residue and one mannose residue, upon which N-glycoproteins are transferred to Golgi apparatus for further processing. **(B)** Processing of N-glycans in Golgi apparatus gives rise to many diverse N-glycan structures bound to asparagine residues (Asn) of N-glycoproteins. They can be broadly be classified into high-mannose, complex and hybrid N-glycans. The illustrations are adapted according to (5,8).

The extent of these modifications is determined by both the structure of the protein and the expression levels of processing enzymes present within GA. These modifications are often species-, tissue- and cell-type-specific, and emerge in the immense diversity of glycosylation patterns, known as microheterogeneity of N-glycoproteins (5,7). Much effort has been made to characterise glycosylation variations of different proteins in both physiological and pathophysiological conditions, but the knowledge on the biological impact of these variations is still fairly limited. Although the exact intrinsic role of heterogeneity of N-glycans is not exactly clear, further characterisation of these differences will provide a better insight on this subject and help translate these discoveries into clinical use.

Another common post-translational modification that tetraspanins acquire on their way through GA is palmitoylation. Palmitoylation is a reversible modification which involves the formation of a covalent thioester

bond between palmitic acid and a cysteine residue of the protein (1). Addition of palmitoyl groups usually occurs on multiple membrane-proximal cysteine residues, conserved in nearly all tetraspanins. This modification has proven to play an important role in tetraspanin protein-protein interactions and is crucial for tetraspanin-enriched microdomains (TEM), a form of specialised membrane platforms important for protein interactions and signalling as discussed below. Thus, palmitoylation of tetraspanins is essential for achieving their biological functions (4).

1.1.3. Tetraspanin functions and interacting partners

Tetraspanins are involved in various cellular processes, including cell motility, adhesion and signalling. They are mainly localized at the plasma membrane, where they function as the molecular organizers of membrane proteins (4,9). Tetraspanins tend to associate with a wide variety of partner proteins, including other tetraspanins, Ig superfamily proteins, proteoglycans, complement regulatory proteins, transmembrane receptors and signalling molecules (10). Although they do not carry out typical receptor-ligand interactions, tetraspanins are thought to be involved in many important biological processes through the formation of TEMs (4). TEMs are found to be implicated in compartmentalization and clustering of functionally related molecules on the plasma membrane, which allows them to act as platforms for signal transduction, adhesion and membrane fusion events (11).

1.1.4. CD63 protein: a constituent of lysosomes and secreted vesicles

CD63, also known as Lysosome Associated Membrane Protein 3 (LAMP-3), is a ubiquitously expressed glycoprotein (12). This member of the tetraspanin family was initially identified as one of the blood platelet activation markers, named Pltgp40, which subsequently proved to be identical to the stage-specific melanoma-associated antigen ME491 (13).

Unlike most tetraspanins, which tend to reside on the cell surface, CD63 is predominantly localized intracellularly within the membranes of the endosomal-lysosomal system. CD63 is amply present in late endosomes (LEs) and lysosomes as well as storage granules like Weibel-Palade bodies of endothelial cells and various secretory lysosome-related vesicles of hematopoietic cells and the α-granules and dense granules of platelets (12,14,15). While a small portion of CD63 can also be found on the cell surface as a part of its regular intracellular trafficking pathway, a high surface expression can be a sign of a pathological process or a reaction to a certain stimulus (14).

Like other tetraspanins, CD63 consists of a short cytosolic N- and C-terminal regions outlining four hydrophobic TM domains, connected by an intracellular loop and two EC loops. EC1 is composed of 19 amino acids with no glycosylation sites. Currently, there is no monoclonal antibody available that recognises the EC1 domain of CD63. (1) EC2 domain is 101 amino acids long, and contains six cysteine residues, including both CCG and PXXC motifs. Sequence analysis revealed that the EC2 domain of human CD63 contains three potential N-glycosylation sites, which are known to be glycosylated to a various extent (12).

Another important structural feature of CD63 is the GYVEM sequence, located in the C-terminal cytoplasmic tail of the protein. It contains a tyrosine-based lysosome sorting Tyr-Xaa-Xaa- Ø (YXXØ) motif, where Xaa can be any amino acid and Ø is a bulky hydrophobic amino acid. Although glycine residue preceding YXXØ motif isn't crucial for its function, it improves the efficiency of lysosomal targeting (3).

Once a newly synthesised CD63 undergoes all necessary modifications in the ER and Golgi compartments, it enters the trans-Golgi network (TGN) (16). TGN acts as a major sorting station for all newly synthesized proteins destined for lysosomes, plasma membrane, and secretory vesicles, depending on the sorting signal in the itinerant protein.

The YXXØ motif is recognised by a μ subunit of adaptor protein (AP) complexes, which mediate intracellular trafficking of CD63 (3,16).

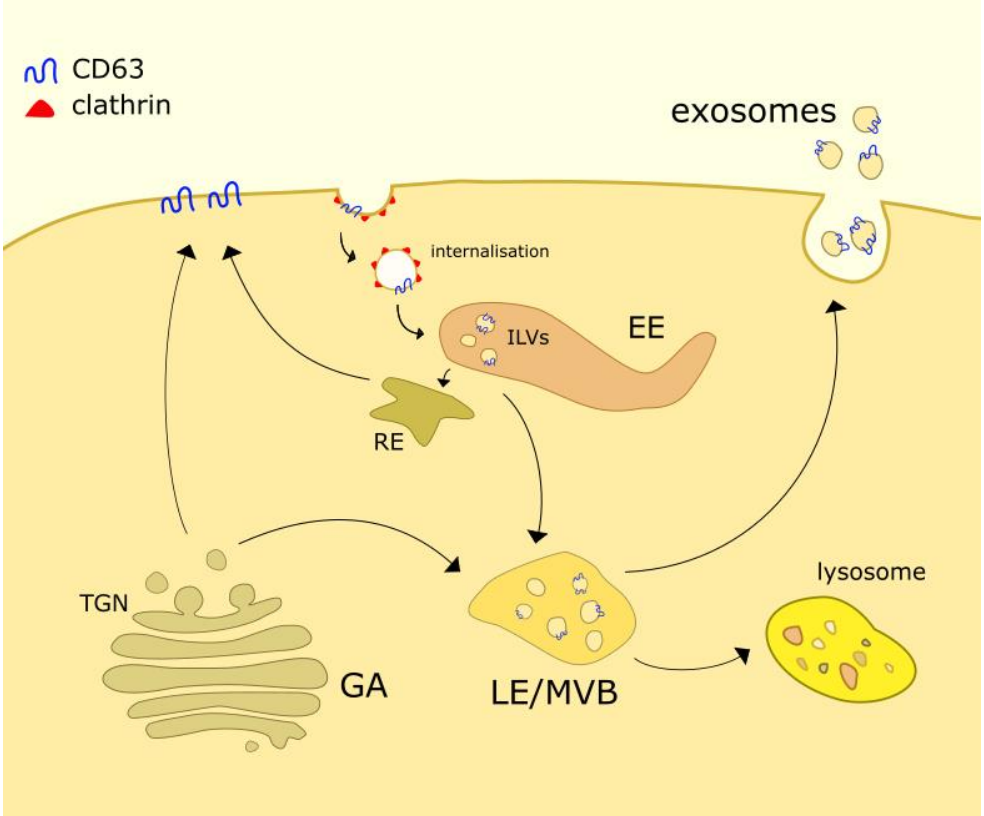


Figure 3. Intracellular trafficking of CD63. Newly synthesised CD63 is transported to late endosomes (LE) /multivesicular bodies (MVB) either directly from trans-Golgi-netwotk (TGN), or indirectly by clathrin-mediated endocytosis from cell surface. Upon internalisation and entering early endosome (EE), CD63 can be incorporated into intraluminal vesicles (ILV) and continue its route to LE/MVB, or it can be transported back to cell surface via recycling endosomes (RE). Once CD63 enters LE/MVB, it can be directed either to lysosomes or secreted within exosomes. The illustration is adapted according to (16).

As depicted in Figure 3, CD63 can be transported to its final destination either through a direct or an indirect pathway. In the direct route, CD63 gets packed in the vesicular carriers that transfer CD63 straight from TGN to LEs (16). Alternatively, CD63 can also be transported from TGN to the cell surface and enter lysosomes indirectly via the endocytic pathway. CD63 is internalised from the plasma membrane by clathrin-mediated endocytosis and subsequently delivered to early endosomes. Afterwards, CD63 can be recycled back to the plasma membrane via recycling endosomes, or directed towards LEs (16,17).

CD63 has been linked with many interaction partners, including integrins (α3β1, α4β1, α6β1), kinases (phosphatidylinositol 4-kinase, Src

kinases Lyn and Hck) and cell surface receptors (CXCR4, MHCII, FcεRI, CD3) (16). Although its exact biological function remains unknown, it has been proposed that CD63 is implicated in signal transduction, cell adhesion and motility. Moreover, CD63 has been shown to participate in the biogenesis of lysosome-related organelles and maturation of melanocytes (18). Despite its ubiquitous expression, a detailed analysis of CD63-deficient mice revealed only a mild phenotype with abnormal renal morphology and altered water balance. This suggests that other proteins with similar expression and structure efficiently compensate for CD63 deficiency in other tissues (19).

1.2. Extracellular vesicles

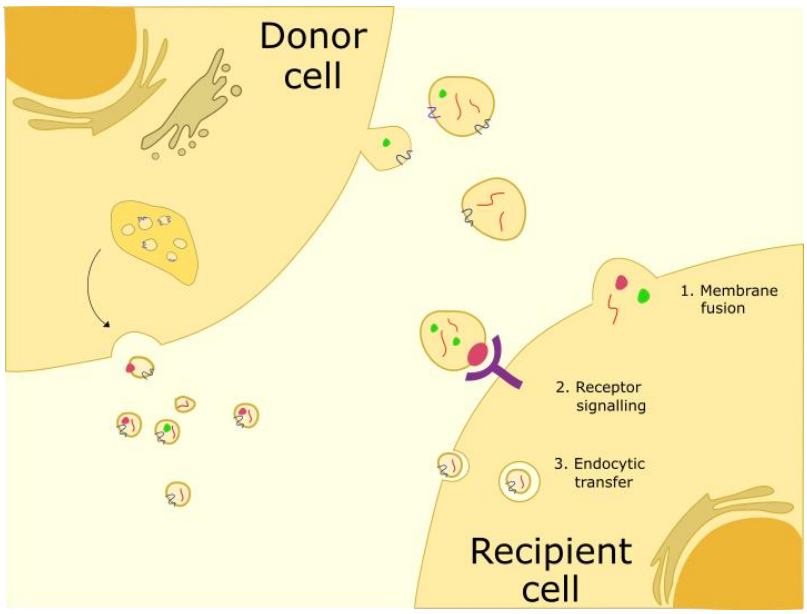
1.2.1. Extracellular vesicles: mediators of intercellular communication

In order to grow and properly perform their biological functions, cells of multicellular organisms need to communicate and work together to provide coordination of cellular events. It is long known that cells can achieve cell-to-cell communication through direct interactions or by secreting various molecules, such as hormones, neurotransmitters and cytokines, into the extracellular space (20). In the last two decades, a new paradigm has emerged that suggests cells can also accomplish intercellular communication through secreting different biomolecules harboured in membrane-enclosed nanoparticles known as extracellular vesicles (EVs) (21).

EVs are a highly heterogeneous population of vesicles, characterized by the presence of phospholipid bilayer surrounding proteins, nucleic acids, lipids and metabolites of the cells they originate from (22). Due to their capability to transfer biological molecules, the area of understanding the biological role of EVs has gained considerable interest from the scientific community. Besides eliminating unwanted materials from the cells, a

growing body of evidence suggests that EVs also participate in the transfer of signalling molecules between physically distant cells (20,22).

In multicellular organisms, EVs are abundantly present in body fluids, where they freely circulate throughout the body (21). Once released, EVs can dock on the surface of a recipient cell, and modify its behaviour by transmitting signals from the cell surface through receptor signalling and/or by releasing their cargo into the cytoplasm of the cell by the membrane fusion or through EV endocytic transfer (Figure 4) (23). It has been proposed that EVs participate in diverse biological processes, ranging from maintenance of cellular homeostasis to promoting the progression of various pathophysiological conditions (21). Despite the huge progress made in the field of EV biology in recent years, knowledge about EV functions and their mechanisms *in vivo* is scarce due to low reproducibility and technical challenges regarding isolation procedures and methods of the characterisation of EV subgroups (22,24).



1.2.2. EV classification and biogenesis

Figure 4. Mechanisms of EV-mediated intercellular communication. EVs can evoke a response from the recipient cell by transmitting signals from the cell surface (receptor signalling), or by releasing their cargo to the cytoplasm of the recipient cell (through membrane fusion or endocytic transfer). The illustration is adapted according to (23).

To date, there have been multiple attempts to classify EVs according to their size, density, protein and lipid composition. However, due to

significant overlapping, these properties proved to be insufficient for a clear distinction between different EV subtypes. Although the consensus on this issue is still not achieved, EVs are most commonly classified based on the mode of their biogenesis. Accordingly, EVs are divided into three main subtypes: exosomes, microvesicles and apoptotic bodies, as depicted in Figure 5 (25,26).

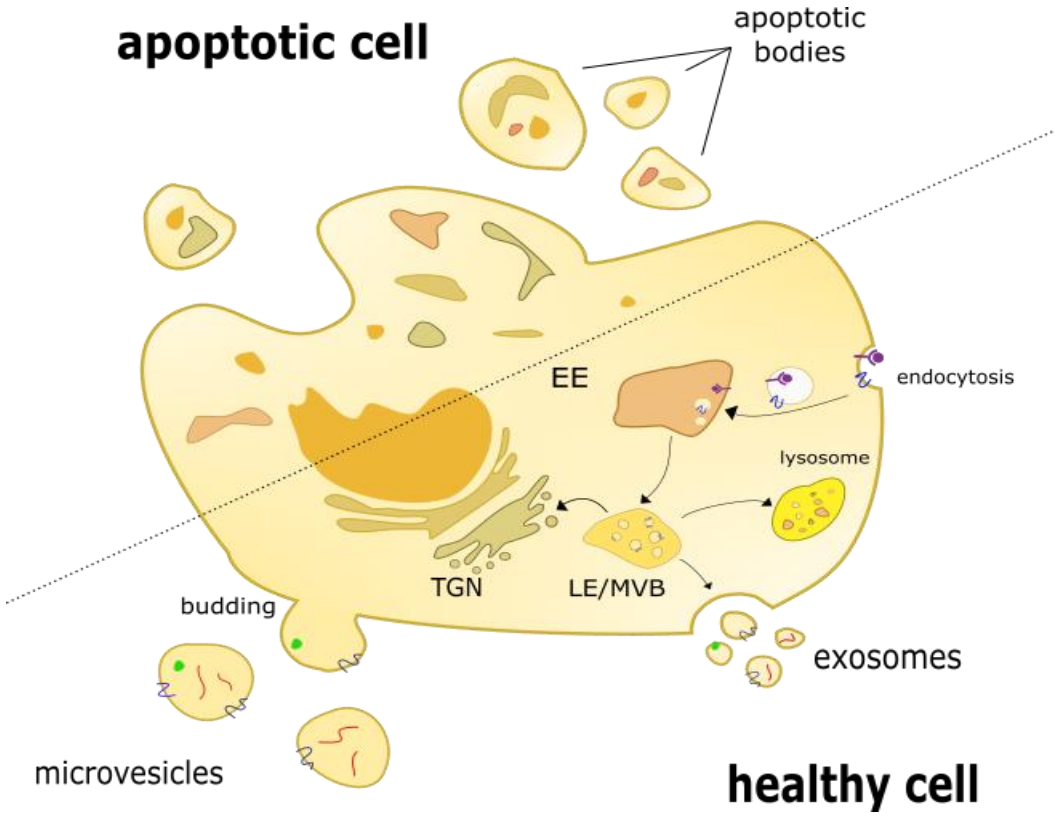


Figure 5. Schematic representation of EV biogenesis pathways. Exosomes are formed within the endosomal system. Following internalisation through endocytosis, cargo is packed within intraluminal vesicles (ILV) by inward invagination of early endosomal (EE) membrane, after which vacuolar regions of EE detach and form of late endosomes/multivesicular bodies (LE/MVB) that can fuse with plasma membrane and release exosomes into extracellular space. Otherwise, LE/MVB can deliver their cargo to lysosomes or direct it towards trans-Golgi network (TGN). Microvesicles are secreted by outward budding and fission of the plasma membrane. Unlike exosomes and microvesicles, which are secreted by healthy cells, apoptotic bodies are generated exclusively by apoptotic cells. The illustration is adapted according to (24,27).

Exosomes represent the smallest type of extracellular vesicles, with the size ranging from 30 to 150 nm in diameter (25). Despite the small size, exosome biogenesis is a surprisingly complex biological process that takes place within the endosomal system of the cell. The endosomal system consists of a series of dynamic and interconnected membrane-bound compartments involved in intracellular transport of cargo molecules

internalized from the cell surface (27). Upon internalization through endocytosis, cargo molecules are delivered to early endosomes (EE) through endocytic vesicles. EE act as a primary sorting station that can recycle cargo molecules back to the plasma membrane via recycling endosomes, or sort them within intraluminal vesicles (ILVs) through a process of inward invagination of the endosomal membrane. As the number of ILV increases, vacuolar regions containing ILVs detach from the rest of EE and form LE/multivesicular bodies (MVB) (27,28). Once formed, LEs/MVBs can deliver ILV cargo to lysosomes, travel towards TGN, or fuse with the plasma membrane and release ILVs as exosomes in the extracellular space (28).

There are still some uncertainties regarding the mechanism of cargo trafficking from endosomes to lysosomes. Some authors advocate the maturation model, which suggests that early endosomes gradually mature into LEs and lysosomes, while others believe that cargo trafficking occurs trough a vesicular transport between these organelles (27,28). Nevertheless, a consensus has not yet been reached on this issue.

Microvesicles, also referred to as shedding vesicles, ectosomes and microparticles, are significantly larger than exosomes, with the size typically ranging from 100 nm to 1 μm in diameter (25). Microvesicles show a distinctive mode of secretion, characterised by a direct outward budding and fission of the plasma membrane. This process is accompanied by localized changes of plasma membrane components, as well as cytoskeletal remodelling and selective recruitment of the cargo which is packed within nascent microvesicles (29).

Apoptotic bodies are the largest EV subtype, with the size varying from 50 nm to 5 μm in diameter (25). Unlike exosomes and microvesicles, which are secreted during normal processes in living cells, apoptotic bodies are generated exclusively by dying cells undergoing apoptosis. During the final phase of apoptosis, the cell starts to disintegrate and fragment into apoptotic bodies, which carry “eat me” signals that trigger phagocytic

uptake by macrophages and other adjacent phagocytic cells. Formation and subsequent phagocytosis of apoptotic bodies prevent spillage of the intracellular contents of the dying cell and enable recycling of apoptotic cell material (30).

1.2.3. Prospects and challenges in clinical applications of EVs

The molecular composition of EV cargo is highly dynamic and depends on the biological status of the parent cell. Accumulating evidence suggests that certain pathological conditions significantly alter the quantity of secreted EVs, as well as the composition of their cargo. For instance, cancer cells have been found to secrete large quantities of EVs that can influence non-malignant cells and promote neo-angiogenesis, invasion and formation of distant metastasis (31). Cancer-derived EVs can carry different cancer-associated molecules, including oncogenic mRNAs, miRNAs and DNA fragments, which makes them a promising source for cancer biomarkers. Similarly, EVs have been shown to carry distinctive biomolecules associated with other diseases, including neurodegenerative diseases, metabolic diseases and autoimmune disorders (22).

One of the main advantages of the utilisation of EVs as diagnostic and prognostic tools is their availability from body fluids. So far, EVs have been found in many body fluids, including blood, cerebrospinal fluid, urine, saliva, synovial fluid, semen, breast milk and amniotic fluid (Figure 6) (32). This makes them an ideal candidate for liquid biopsies. In contrast to standard tissue biopsies, a liquid biopsy presents a minimally- or even non-invasive diagnostic technique for early disease detection, assessing treatment response and detection of disease recurrence (31). This approach also offers a valuable alternative for monitoring diseases affecting tissues and organs that are otherwise inaccessible or when the diagnostic procedure poses a significant risk to a patient (32). Still, multiple issues have yet to be resolved before EV clinical translation.

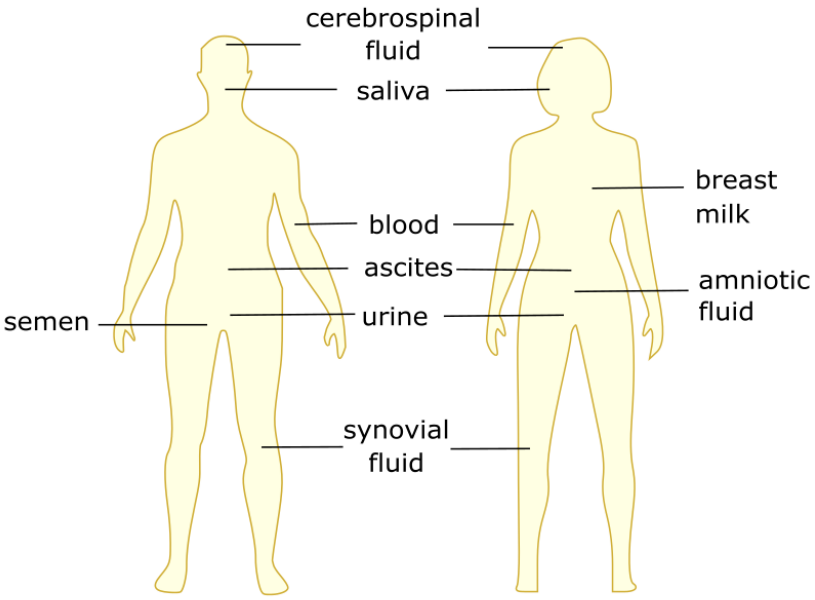


Figure 6. EVs can be found in various body fluids. So far, EVs have been isolated from most body fluids, including amniotic fluid, milk, blood, cerebrospinal fluid, saliva, semen, synovial fluid and urine. The illustration is adapted according to (34).

The first step in any EV research is choosing the optimal EV isolation strategy. Due to the high complexity of body fluids, separation of EVs from other non-EV particles with sufficient yields and purity can present an extremely formidable task (22). The most widely used methods of EV isolation currently include ultracentrifugation, either of differential or density gradient type, immunoaffinity capture, size-exclusion chromatography and microfluidic technology (32). However, there is still no standardised protocol for EV isolation, which hinders clinical translation of discoveries in the EV field.

Characterisation of isolated EV subtypes presents another major challenge for EV studies. Since exosomes and microvesicles are both commonly found in body fluids, isolation procedures typically yield complex mixtures of these EV populations (25). As a consequence of the substantial overlapping in their size ranges, it is essentially impossible to discriminate between the two based solely on their size (33). Hence, other means of EV subtype characterization are being actively explored.

Much effort has been devoted to identifying specific markers of exosomes and microvesicles. EVs tend to be highly enriched with several transmembrane proteins, including tetraspanins CD9, CD63 and CD81 (3). Although they were originally thought to be specific markers of exosomes, more recent studies revealed that these proteins can also be present in both microvesicles and apoptotic bodies. Nevertheless, tetraspanins can still be appropriate for utilisation in affinity-based isolation and purification methods of EVs from biological samples (3,25).

An additional problem that needs to be considered in EV research is the tissue identification of EV origin. Since EVs isolated from the body fluids present a heterogeneous mixture of vesicles secreted by virtually all tissues, determining the tissue of origin of a single EV or a subset of EVs presents a particularly complicated task (33). Some tissue-specific molecules found within EVs have been described, but their presence in the EV isolates merely indicates that some of the isolated EVs originate from that specific tissue. Thus, selective isolation of EV populations originating from specific cell types and/or characterisation of EVs at a level of a single vesicle could be beneficial in the utilisation of EVs for diagnostic purposes and further clinical applications (32). Identification of a tissue-specific EV marker could also help in addressing this issue, but such a marker has not yet been described.

2. Aims of the thesis

The aim of this study was to characterise a novel mouse anti-CD63 antibody mCD63.07 directed against mouse CD63 protein and produced at the Center for Proteomics, University of Rijeka, Faculty of Medicine, Croatia. The preliminary results obtained during initial screening of antibodies indicated that mCD63.07 specifically detects CD63 in western blot analysis.

Thus, the specific objectives of this thesis were:

1) to isolate proteins from the following wild type (wt) and CD63 knockout (ko) tissues: salivary gland, brain, lung, liver, kidney, muscle, bone marrow and spleen;
2) to analyse tissue expression of CD63 by western blot analysis;
3) to determine the antibody specificity by comparing signal of corresponding protein lysates of wt and ko tissues;
4) to investigate whether the tissue-specificity of CD63 forms arises from differential N-glycosylation of the CD63 by performing a deglycosylation treatment of mouse wt tissue lysates, followed by an immuno blot analysis of the samples.
5) if the antibody specificity is proven then to perform alignment of mouse and human EC2 domain of CD63

3. Materials and methods

3.1. Antibody production

The mouse monoclonal mCD63.07 antibody was produced and purified by collaborators at the Center for Proteomics, Faculty of Medicine in Rijeka, and these procedures are not part of the diploma thesis. However, main features of the mCD63.07 production are described to provide basic information about antigen and immunisation and thereby to enable better insight into antibody properties.

Briefly, the nucleotide sequence encoding the EC2 domain of CD63 protein was cloned into the pFUSE-hIgG1-Fc2 plasmid to create a fusion protein with human IgG1-Fc region and an IL2 signal sequence (IL2ss) (34). The pFUSE-hIgG1-Fc2-CD63 plasmid was transiently transfected into HEK293T cells which secreted recombinant protein into the supernatant thanks to the IL2ss part of the fusion protein. The supernatant was collected, and recombinant proteins were purified by affinity chromatography based on hIgG1-Fc (Figure 7).

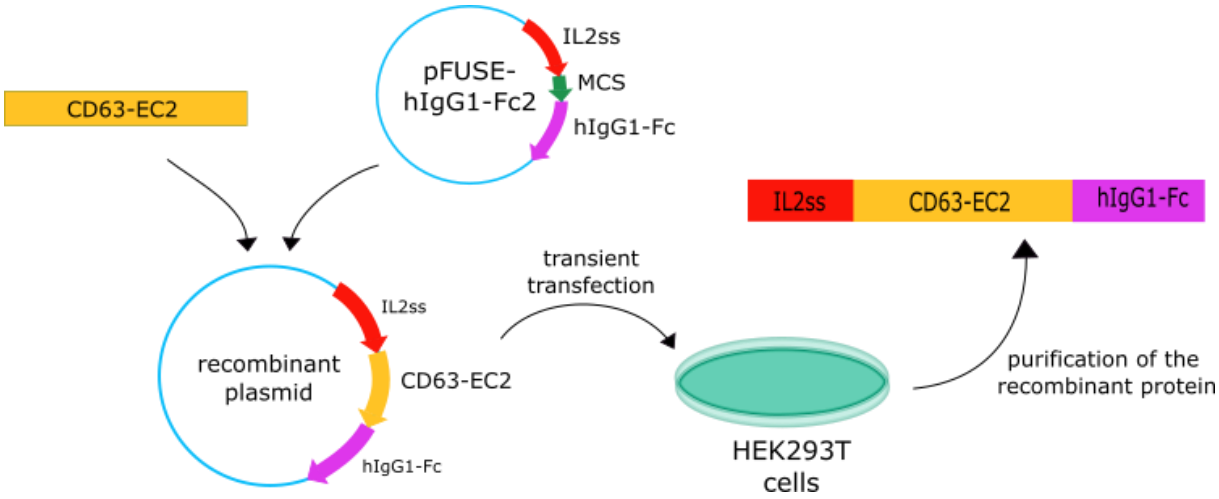


Figure 7. Simplified scheme of production of recombinant CD63 protein used for mouse immunisation to generate anti-CD63 antibody. Nucleotide sequence encoding EC2 domain of the CD63 protein (CD63-EC2) was cloned into multiple cloning site (MCS) of the pFUSE-hIgG1-Fc2 plasmid, situated between interleukin 2 signal sequence (IL2ss) and human IgG1 Fc sequence (hIgG1-Fc). HEK293T cells were transiently transfected with the recombinant plasmid, and the recombinant protein was collected and purified from the supernatant of the transfected cells.

The purified recombinant protein containing CD63-EC2 was used to immunise CD63-knockout mice (19) and also to screen serum and supernatant antibodies by ELISA. Mice with high titres of serum anti-CD63 antibodies were used for making the monoclonal antibodies applying standard protocols for hybridoma production. The mCD63.07 was cloned from a stable and well productive mother well and was shown to be of mouse IgG2b isotype.

3.2. Isolation of mouse tissues

All procedures involving animals in this study were performed in accordance with local law requirements. CD63-knockout and C57BL/J6 mice were euthanised by CO_2 aspiration followed by cervical dislocation, performed by a qualified person and in the accordingly certified facility at the Department of Histology - Laboratory Mouse Breeding and Engineering Centre (LAMRI), University of Rijeka, Faculty of Medicine. The corresponding statement issued by authorised officers is provided as annex in Section 9.

Afterwards, animals were disinfected with 70% ethanol and promptly dissected. Tissue samples of the salivary gland, brain, lung, liver, kidney, muscle, bone marrow and spleen were collected and placed in individual 2 ml tubes on ice. Obtained tissue samples were rapidly frozen using snap freeze method in liquid nitrogen, and stored at -80 °C.

3.3. Preparation of protein lysates from mouse tissues

Proteins were extracted from tissue samples by homogenization in 0.5-0.7 ml RIPA lysis buffer (Thermofisher, 25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP40, 1% deoxycholate, 0.1% SDS) with the addition of protease inhibitor (Roche Diagnostics GmbH). Tissue samples were incubated with the lysis buffer on ice for 30 minutes, after which the samples were centrifuged (Eppendorf) at 16.000xg and 4 °C for 30 minutes. Protein-

containing supernatant was collected and stored in the individual tubes at -80 °C. Total protein concentration of the lysates was determined by the Bradford protein assay. Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories) was diluted with demineralized water in 1:5 ratio and used to dissolve up to 2 µl of protein lysate. The absorbance was read at 595 nm (Beckman Coulter DU730 UV-Vis Spectrophotometer) and the protein concentration was calculated using the standard curve obtained by bovine serum albumin dilutions of known concentration. Protein samples were diluted to a concentration of 0.5 µg/µl with 5x Laemmli buffer (1M Tri HCl pH 6.8, 50% glycerol (v/v), 10% SDS (w/v), 0.05% bromophenol blue (w/v), 2-mercaptoethanol) and distilled water. Prepared lysates were boiled for 5 minutes at 95 °C on the heating block, spun down at 4 °C in a microcentrifuge for a few seconds and stored at -20 °C.

3.4. Peptide-N-glycosidase F digestion

Protein deglycosylation was performed on wt tissue lysates with peptide-N-glycosidase F (PNGase F, New England Biolabs) according to the producer's instructions. Briefly, for the initial denaturation step, a volume containing 2 µg (for salivary gland, bone marrow and kidney sample) or 15 µg (for liver, muscle and spleen sample) was combined with 1 µl of 10x Glycoprotein denaturing buffer and filled up with distilled water to make a total of 10 µl of reaction volume. The denaturation reaction was incubated for 10 minutes at 100 °C on the heating block. PNGase F mastermix was prepared by combining 22 µl of 10x Glycobuffer 2, 22 µl of 10% NP-40 (Sigma-Aldrich), 1 µl of PNGase F (500 U/µl) and 66 µl of distilled water. Denatured glycoproteins were cooled down on ice, centrifuged for 10 seconds and combined with 10 µl of PNGase F mastermix. The reaction was incubated for 1 hour and 45 minutes at 37 °C. Once the reaction was finished, 5 µl of 5x Laemmli buffer was added to each sample, samples were boiled for 5 minutes at 95 °C on the heating block, spun down at 4 °C in a microcentrifuge (Eppendorf) for a few seconds and stored at -20 °C.

3.5. Protein electrophoresis and western blot analysis

Up to 10 µg of proteins per lane were separated by SDS-PAGE on 12% polyacrylamide gel. SDS-PAGE was performed in 1x electrophoresis running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) using vertical protein electrophoresis chamber (Mini-PROTEAN, Bio-Rad). Proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore) in 1x transfer buffer (25 mM Tris, 192 mM Glycine, 20% methanol) using transfer apparatus (Mini Trans-Blot® Cell, Bio-Rad) filled with 1x transfer buffer. A reusable ice pack and a magnet were also placed inside the tank to reduce the excessive heating of the buffer during the transfer. The tank was placed on the magnetic stirrer and the transfer was performed at 70 V for 1.5 hr. Transfer efficiency was determined by staining PVDF membranes with Ponceau S (0.1% Ponceau S in 5% acetic acid). To prevent the non-specific binding, the membrane was blocked by incubation with 5% milk (non-fat dry milk, Santa Cruz) /TBS (20 mM Tris, 150 mM NaCl) blocking buffer for 5 minutes at room temperature. Membranes were then incubated with either mCD63.07 (lot_001, 7 ug/ml in 5% milk/TBS; Center for Proteomics, University of Rijeka, Faculty of Medicine, Croatia) or mouse anti-pan-actin primary antibody (diluted 1:80.000 in 5% milk/TBS; clone 2A3, Millipore) in 5% milk/TBS overnight on the rocking table at 4 °C. On the following day, membranes were washed in 1xTBST (20 mM Tris, 150 mM NaCl, 0.05% Tween-20) 3 times for 5 minutes each time, to remove the unbound antibody from the membrane. Membranes were then incubated with secondary anti-mouse antibody (diluted 1:50.000 in 5% milk/TBS; Jackson ImmunoResearch) for 30 min, followed by washing in 1xTBST 3 times for 5 minutes each time. Ultimately, proteins were visualised by using chemiluminescent substrates SignalFire Plus ECL Reagent and SignalFire Elite ECL Reagent (Cell Signaling Technology) and the chemiluminescence was detected using UviTec Alliance 4.7 imaging system.

3.6. Alignment of EC2 domain sequences of mouse and human CD63

FASTA sequences of mouse and human EC2 domain of CD63 were downloaded from Uniprot database (entry P41731: CD63_MOUSE; entry P08962: CD63_HUMAN). Alignment of sequences was generated using Clustal Omega (35) under default parameters. Percent identity of amino acid sequences was calculated using BLAST(36). The predicament of N-glycosylation sites was made using NetNGlyc prediction software (37).

4. Results

4.1. mCD63.07 specifically detects murine CD63 protein *in vivo*

To test if the novel mCD63.07 antibody binds to CD63 in a specific manner, western blot analysis was performed on wt and CD63 ko murine tissue samples. The mCD63.07 antibody detected a series of specific bands in the range of 35-130 kDa which were present in wt samples, but absent in the ko samples while actin was detected at comparable levels in all corresponding wt and ko tissues (Figure 8). However, an unspecific band at approximately 50 kDa appeared in salivary gland and spleen CD63 ko tissue samples.

Next to the demonstrated specificity, mCD63.07 was able to detect highly varying levels of CD63 protein in analysed wt tissues. Salivary gland, brain, lung, kidney, bone marrow tissue samples exhibited high level of CD63 protein, whilst low CD63 expression was detected in spleen tissue. Wt liver tissue sample reveals only a single band at approximately 50 kDa that appears to be non-specific, while no signal was detected in muscle tissue samples at this protein loading.

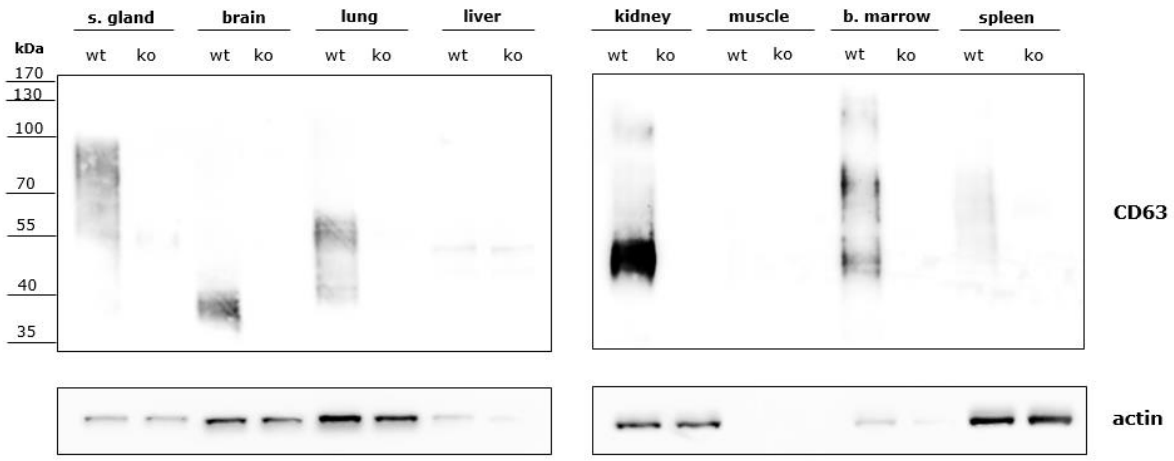


Figure 8. mCD63.07 specifically recognises CD63 protein *in vivo*. Protein samples of wild type (wt) and CD63-knockout (ko) salivary gland, brain, lung, liver, kidney, muscle, bone marrow and spleen tissue were analysed by immunoblot using the newly produced mouse monoclonal antibody mCD63.07. Actin was used as a loading control for all samples. Shown are representative images of 3 experiments.

4.2. Murine tissues significantly differ in CD63 protein forms

To further characterize different forms of CD63 protein present in analysed tissues, additional immunoblot analyses were performed with better protein separation and increased protein loading (from the initial 5 µg of total protein load to up to 10 µg of total protein load, depending on the tissue) for tissues with low level of CD63 expression (Figure 9). CD63 detected in wt salivary gland tissue appeared as a smear ranging from 25 to 100 kDa. Just like in the previous experiment, a single band at approximately 50 kDa was detected in CD63 ko salivary gland tissue. Wt brain tissue revealed a much narrower span of bands, ranging from 35 to 50 kDa, with the strongest signal between 37-40 kDa, while wt lung tissue exhibited a strong signal between 38 and 55 kDa. CD63 was not detected in brain and lung CD63 ko tissue samples. Wt spleen tissue sample appeared as a smear ranging from 36 to 55 kDa, with bands at approximately 25, 27 and 50 kDa. No signal was detected for CD63 ko spleen sample. CD63 detected in wt kidney tissue appeared as a smear ranging from 39 to 50 kDa, with fainter protein laddering 50 to 110 kDa and a single weak band at 35 kDa, while no bands were detected in CD63 ko lung tissue. Wt muscle tissue revealed a very weak signal between 36 and 40 kDa, while bone marrow exhibited a wide span of bands ranging from 36 to 170 kDa and a single band at 25 kDa. No signal was detected in muscle and bone marrow CD63 ko tissues.

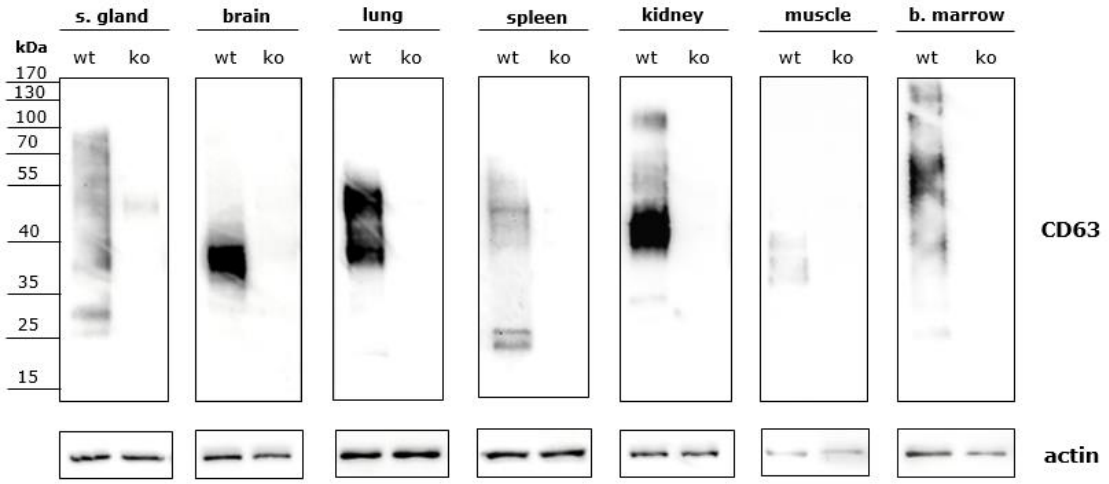


Figure 9. Forms of CD63 expressed in different mouse tissues are tissue-specific. Protein samples of wild type (wt) and CD63-knockout (ko) salivary gland, brain, lung, spleen, kidney, muscle and bone marrow tissue were analysed by immunoblot using the newly produced mouse monoclonal antibody mCD63.07. Actin was used as a loading control for all samples.

4.3. mCD63.07 binds to both glycosylated and deglycosylated CD63 protein

N-glycosylation has been recognised as the main post-translational modification of the CD63 protein along with palmitoylation (12). To investigate whether the observed tissue-specificity of CD63 forms in different murine tissues originates from differential N-glycosylation of the protein, deglycosylation treatment was performed on wt murine tissue samples. All wt tissue samples were subjected to digestion with PNGase F, followed by immunoblot analysis with mCD63.07 antibody (Figure 10). The treatment of salivary gland, kidney, bone marrow and spleen tissue resulted in the complete fusion of higher molecular weight bands into one band at approximately 24 kDa. A strong band at 24 kDa was also detected in liver and spleen tissues, but some higher molecular weight bands were also present in the samples after the treatment. CD63 detected in the digested liver sample appeared as a very faint series of bands ranging from 40 to 70 kDa, and a stronger band at 24 kDa. Treated muscle sample exhibited a much stronger signal at 24 kDa, but an additional band was also visible just below 55 kDa.

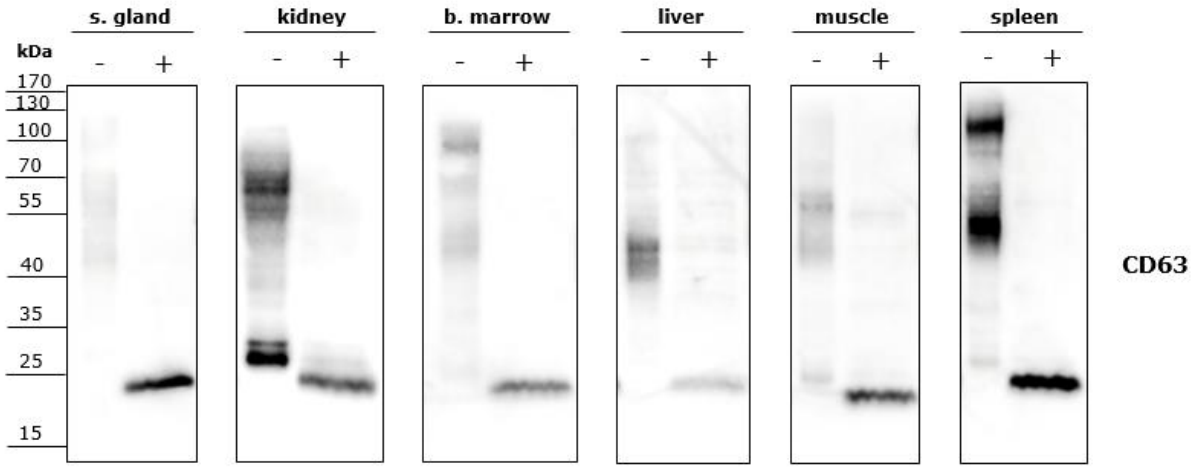


Figure 10. Deglycosylation treatment of murine tissue lysates with PNGase F causes fusion of higher molecular weight bands into one band at approximately 24 kDa. Tissue samples with high CD63 content (salivary gland, kidney and bone marrow) and low CD63 content (liver, muscle and spleen) were used at 2 µg or 15 µg of proteins, respectively for digestion by PNGase F (+). Corresponding protein amounts without PNGase F treatment were loaded in parallel (-). Proteins were separated by SDS-PAGE and analysed by immunoblot using the newly produced mouse monoclonal antibody mCD63.07.

4.4. Mouse and human EC2 domains of CD63 protein share high sequence similarity

Since EC2 domain was used as antigen for the mCD63.07 antibody production, an epitope can only be placed somewhere inside the EC2 domain of the CD63 protein (Figure 11A, amino acids 103-203). To assess potential cross-reactivity of mCD63.07 antibody with human CD63 protein, sequences of EC2 domain of the mouse and human CD63 orthologs were aligned and analysed (Figure 11B). Human and mouse EC2 sequences were compared using BLAST, which calculated 100% query coverage, 83,17% sequence similarity and 67,33% sequence identity. In addition, predicament of potential N-glycosylation showed that mouse and human CD63 share three common potential N-glycosylation sites N130, N150 and N172, while the mouse orthologue contained an additional N-glycosylation site N116.

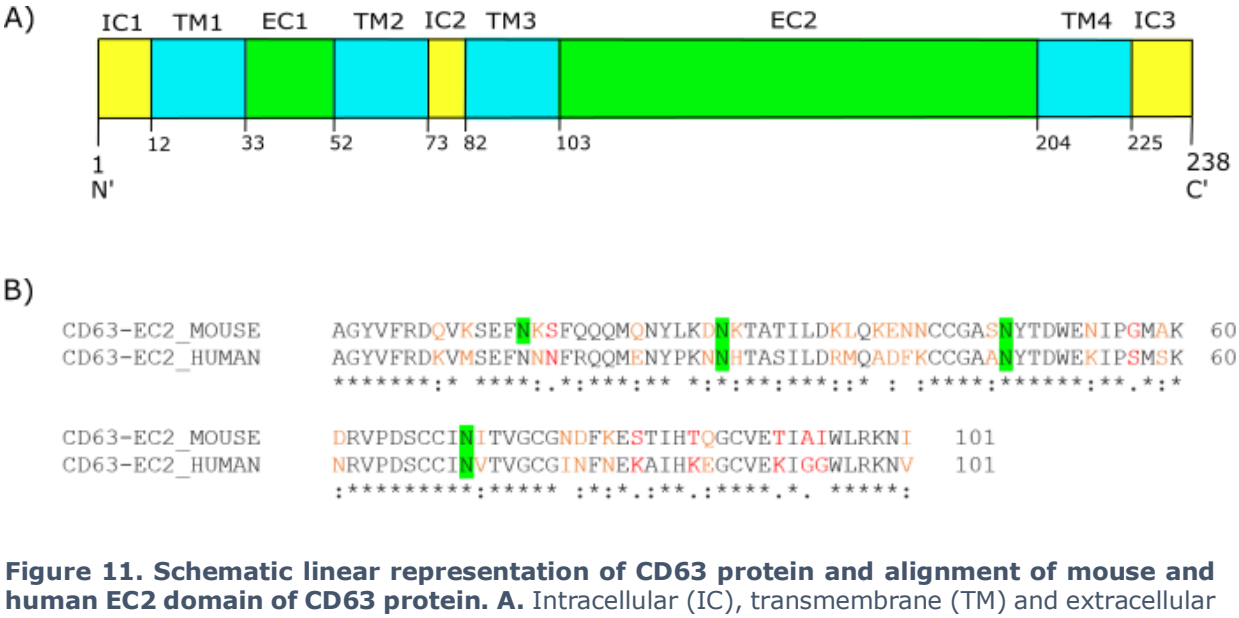


Figure 11. Schematic linear representation of CD63 protein and alignment of mouse and human EC2 domain of CD63 protein. A. Intracellular (IC), transmembrane (TM) and extracellular (EC) domains are colour-coded on primary sequence of CD63 with denoted aminoacid positions. **B.** FASTA sequences were downloaded from the Uniprot database and aligned using Clustalo Omega Multiple Sequence Alignment tool. "*" indicates perfect alignment (black); ":" indicates a site belonging to group exhibiting strong similarity (orange); "." indicates a site belonging to a group exhibiting weak similarity (red); potential N-glycosylation sites are highlighted in green colour.

5. Discussion

CD63 is a glycosylated tetraspanin, frequently used as a protein marker for EVs (3). Additionally, CD63 has been recognised as an activation marker of various cell types, including basophils, mast cells and platelets (12). Measuring of surface expression of CD63 in basophils has proven to be particularly useful, and is being employed in basophil activation test for diagnosing allergies (38). While other biological implications of CD63 are being actively explored, the molecular role of CD63 is still poorly understood. Currently, there is no commercially available knockout-validated mouse CD63 antibody that is characterised as being able to recognise different glycosylated forms of CD63. This study describes that the novel mouse monoclonal antibody mCD63.07 produced at the Center of Proteomics, Faculty of Medicine in Rijeka, possesses such properties and might contribute to further characterisation of CD63.

The initial objective of this study was to investigate the specificity of the mouse mCD63.07 antibody and to examine the forms of CD63 found in different mouse tissues using immunoblot analysis. The results demonstrated that the mCD63.07 antibody specifically recognises different CD63 forms, which were not present in the CD63-knockout tissue samples, thus validating the antibody's specificity. A nonspecific signal was observed in CD63-knockout salivary gland and liver samples at approximately 50 kDa. It is highly plausible that the nonspecific signal originates from the binding of the secondary anti-mouse antibody to the heavy chain of the endogenous murine immunoglobulins naturally occurring in the mouse tissue.

Although multiple studies observed CD63 expression in cell cultures, data about CD63 expression *in vivo* is limited. Kennel et al. reported that the CD63 protein is expressed in several rat tissues, including kidney, spleen and salivary gland (39). Immunohistochemical analysis of CD63 protein on human tissue sections revealed several other tissues that

express high levels of CD63, including liver, lung, cerebral cortex and bone marrow mononuclear cells (40). In this study, various levels of CD63 were detected in brain, kidney, lung, salivary gland, bone marrow, muscle, spleen, and liver tissue. The strongest expression was observed in kidney, brain, and lung tissue, which correlates to previous findings of CD63 expression (19,39,40). Tissue-specific roles of CD63 protein were described in CD63 deficient mice which suffered of kidney pathology and altered water balance indicating the high significance of CD63 for kidney function (19). As reported by Schultze et al., CD63 plays a role in basolateral sorting and membrane distribution of organic cation transporter 2 protein in renal proximal tubules (41). Apart from its function in renal proximal tubules, it has been reported that CD63 is also involved in platelet adhesion and spreading as well as the maturation of melanocytes (18,42). These findings suggest that the CD63 protein participates in various specialized functions of different cell types. The novel mCD63.07 antibody might contribute to better characterisation of these specialised CD63 forms due to antibody's applicability in immunoblot technique and thus ability to detect CD63 forms of different sizes.

Previous studies mostly reported CD63 as a 30-65 kDa protein (12). Whilst some researchers reported the presence of CD63 with molecular a mass higher than 65 kDa, the possibility of tissue-specific distribution of different CD63 forms has not yet been addressed. Novel mCD63.07 antibody recognised multiple CD63 forms, ranging from 25 to 170 kDa, which differed among the examined tissues. While some tissues, including brain, lung and liver, expressed forms of CD63 with lower molecular mass (35-55 kDa), others produced a much broader range of CD63 forms, extending up to 170 kDa. Even though some forms of CD63 appear to be common in most tissues (mainly in 35-60 kDa range), others appear to be tissue-specific. Identification of tissue-specific forms of CD63 could prove beneficial in developing new diagnostic methods involving EVs. Since CD63 is already recognised as a marker of EVs and is being used for EV isolation, recognising tissue-specific forms of this protein might help solve the

problem regarding identification of EV tissue of origin. Further characterisation of these tissue-specific forms of CD63 using immunoprecipitation combined with mass spectroscopy analysis could provide a better insight into the structure of the glycan portion of CD63.

This study also showed that digestion of wild type tissue samples with PNGase F resulted in merging of bands with higher molecular weight into one band with a molecular weight of 24 kDa, which corresponds to predicted molecular mass of the protein itself. The result indicates that the observed differences in molecular weight between molecular forms of CD63 exist due to differences in the structure of carbohydrates attached to CD63. Novel mCD63.07 antibody was able to bind both glycosylated and deglycosylated forms of murine CD63, which indicates that mCD63.07 antibody recognises the protein portion of CD63. Since the tissue-specific expression of CD63 glycoforms has been observed, it is also possible that different forms of CD63 engage in different intracellular processes. However, further research will be required to address these speculations.

Due to the high specificity and sensitivity of mCD63.07 antibody demonstrated on murine tissues, a cross-reactivity to the human CD63 would present an added value for studying the CD63 biology. Since the testing of mCD63.07 antibody cross-reactivity was beyond the scope of this study, an alignment of the EC2 domain sequences was performed instead. The EC2 domain of human CD63 shares a relatively high sequence similarity (84%) and sequence identity (67%) with mouse CD63 orthologue, but since the exact epitope of the antibody is not known, it is not possible to accurately predict if the antibody will bind to human CD63. If the antibody proves to be cross-reactive with human CD63, it will broaden its applicability and allow characterisation of human CD63 glycoforms.

6. Conclusion

The results of this study show that the novel mCD63.07 antibody recognises both glycosylated and non-glycosylated forms of the murine CD63 protein. Immunoblot analysis of the wild type and corresponding CD63-knockout murine tissues demonstrated that the novel antibody specifically recognises a linear epitope on the murine CD63.

Comparison of the signal obtained in different mouse tissues revealed that mCD63.07 antibody binds different glycoforms of CD63, some of which appear to be tissue-specific.

Since CD63 is already being used as a marker of EVs, identifying tissue-specific forms of this EV marker protein could widen its applicability and provide new methods for identification of EV tissue of origin. To further analyse its utility, the antibody should be tested for cross-reactivity with human CD63. Future CD63 research should include better characterisation of CD63 glycoforms, possibly by immunoprecipitation combined with mass spectrometry. This approach would allow the identification of specific CD3 glycoforms, which could be useful for EV-related diagnostics.

7. References

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

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 Sep;5:442.

4. Termini CM, Gillette JM. Tetraspanins Function as Regulators of Cellular Signaling. Front Cell Dev Biol. 2017;5:34.

5. Bieberich E. Synthesis, Processing, and Function of N-glycans in N-glycoproteins. Adv Neurobiol. 2014 Aug;9:47–70.

6. Schwarz F, Aebi M. Mechanisms and principles of N-linked protein glycosylation. Curr Opin Struct Biol. 2011 Oct;21(5):576–82.

7. Hossler P, Mulukutla BC, Hu W-S. Systems analysis of N-glycan processing in mammalian cells. PloS One. 2007 Aug;2(8):e713.

8. Higel F, Seidl A, Sörgel F, Friess W. N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins. Eur J Pharm Biopharm Off J Arbeitsgemeinschaft Pharm Verfahrenstechnik EV. 2016 Mar;100:94–100.

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

10. Hemler ME. Specific tetraspanin functions. J Cell Biol. 2001 Dec 24;155(7):1103–8.

11. Yáñez-Mó M, Barreiro O, Gordon-Alonso M, Sala-Valdés M, Sánchez-Madrid F. Tetraspanin-enriched microdomains: a functional unit in cell plasma membranes. Trends Cell Biol. 2009 Sep;19(9):434–46.

12. Metzelaar MJ, Wijngaard PL, Peters PJ, Sixma JJ, Nieuwenhuis HK, Clevers HC. CD63 antigen. A novel lysosomal membrane glycoprotein, cloned by a screening procedure for intracellular antigens in eukaryotic cells. J Biol Chem. 1991 Feb;266(5):3239–45.

13. Azorsa DO, Hyman JA, Hildreth JE. CD63/Pltgp40: a platelet activation antigen identical to the stage-specific, melanoma-associated antigen ME491. Blood. 1991 Jul;78(2):280–4.

14. Kobayashi T, Vischer UM, Rosnoblet C, Lebrand C, Lindsay M, Parton RG, et al. The tetraspanin CD63/lamp3 cycles between endocytic and secretory compartments in human endothelial cells. Mol Biol Cell. 2000 May;11(5):1829–43.

15. 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.

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

17. Peden AA, Oorschot V, Hesser BA, Austin CD, Scheller RH, Klumperman J. Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. J Cell Biol. 2004 Mar;164(7):1065–76.

18. 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;21(4):708–21.

19. 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.

20. Yoon YJ, Kim OY, Gho YS. Extracellular vesicles as emerging intercellular communicasomes. BMB Rep. 2014 Oct;47(10):531–9.

21. Yáñez-Mó M, Siljander PR-M, Andreu Z, Zavec AB, Borràs FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. J Extracell Vesicles. 2015 May;4:27066.

22. Kalra H, Drummen GPC, Mathivanan S. Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. Int J Mol Sci. 2016 Feb;17(2):170.

23. Extracellular vesicle docking at the cellular port: Extracellular vesicle binding and uptake. - PubMed - NCBI [Internet]. [cited 2020 Apr 14]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/28104520

24. Margolis L, Sadovsky Y. The biology of extracellular vesicles: The known unknowns. PLoS Biol. 2019 Jul;17(7):e3000363.

25. Doyle LM, Wang MZ. Overview of Extracellular Vesicles, Their Origin, Composition, Purpose, and Methods for Exosome Isolation and Analysis. Cells. 2019 Jul;8(7).

26. Raposo G, Stoorvogel W. Extracellular vesicles: Exosomes, microvesicles, and friends. J Cell Biol. 2013 Feb;200(4):373–383.

27. Elkin SR, Lakoduk AM, Schmid SL. Endocytic pathways and endosomal trafficking: a primer. Wien Med Wochenschr 1946. 2016 May;166(7–8):196–204.

28. Scott CC, Vacca F, Gruenberg J. Endosome maturation, transport and functions. Semin Cell Dev Biol. 2014 Jul;31:2–10.

29. Tricarico C, Clancy J, D'Souza-Schorey C. Biology and biogenesis of shed microvesicles. Small GTPases. 2017 Feb;8(4):220–32.

30. Xu X, Lai Y, Hua Z-C. Apoptosis and apoptotic body: disease message and therapeutic target potentials. Biosci Rep. 2019 Jan;39(1).

31. Xu R, Rai A, Chen M, Suwakulsiri W, Greening DW, Simpson RJ. Extracellular vesicles in cancer - implications for future improvements in cancer care. Nat Rev Clin Oncol. 2018 Oct;15(10):617–38.

32. Armstrong D, Wildman DE. Extracellular Vesicles and the Promise of Continuous Liquid Biopsies. J Pathol Transl Med. 2018 Jan;52(1):1–8.

33. Ramirez MI, Amorim MG, Gadelha C, Milic I, Welsh JA, Freitas VM, et al. Technical challenges of working with extracellular vesicles. Nanoscale. 2018 Jan;10(3):881–906.

34. Železnjak J, Lisnić VJ, Popović B, Lisnić B, Babić M, Halenius A, et al. The complex of MCMV proteins and MHC class I evades NK cell control and drives the evolution of virus-specific activating Ly49 receptors. J Exp Med. 2019 May;216(8):1809–27.

35. Madeira F, Park YM, Lee J, Buso N, Gur T, Madhusoodanan N, et al. The EMBL-EBI search and sequence analysis tools APIs in 2019. Nucleic Acids Res. 2019 Feb;47(W1):W636–41.

36. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 1997 Sep;25(17):3389–402.

37. Gupta R, Jung E, Brunak S. Prediction of N-glycosylation sites in human proteins. 2004 Jan;46:203–6.

38. Eberlein-König B, Rakoski J, Behrendt H, Ring J. Use of CD63 expression as marker of in vitro basophil activation in identifying the culprit in insect venom allergy. J Investig Allergol Clin Immunol. 2004 Apr;14(1):10–6.

39. Kennel SJ, Lankford PK, Foote LJ, Davis IA. Monoclonal antibody to rat CD63 detects different molecular forms in rat tissue. Hybridoma. 1998 Dec;17(6):509–15.

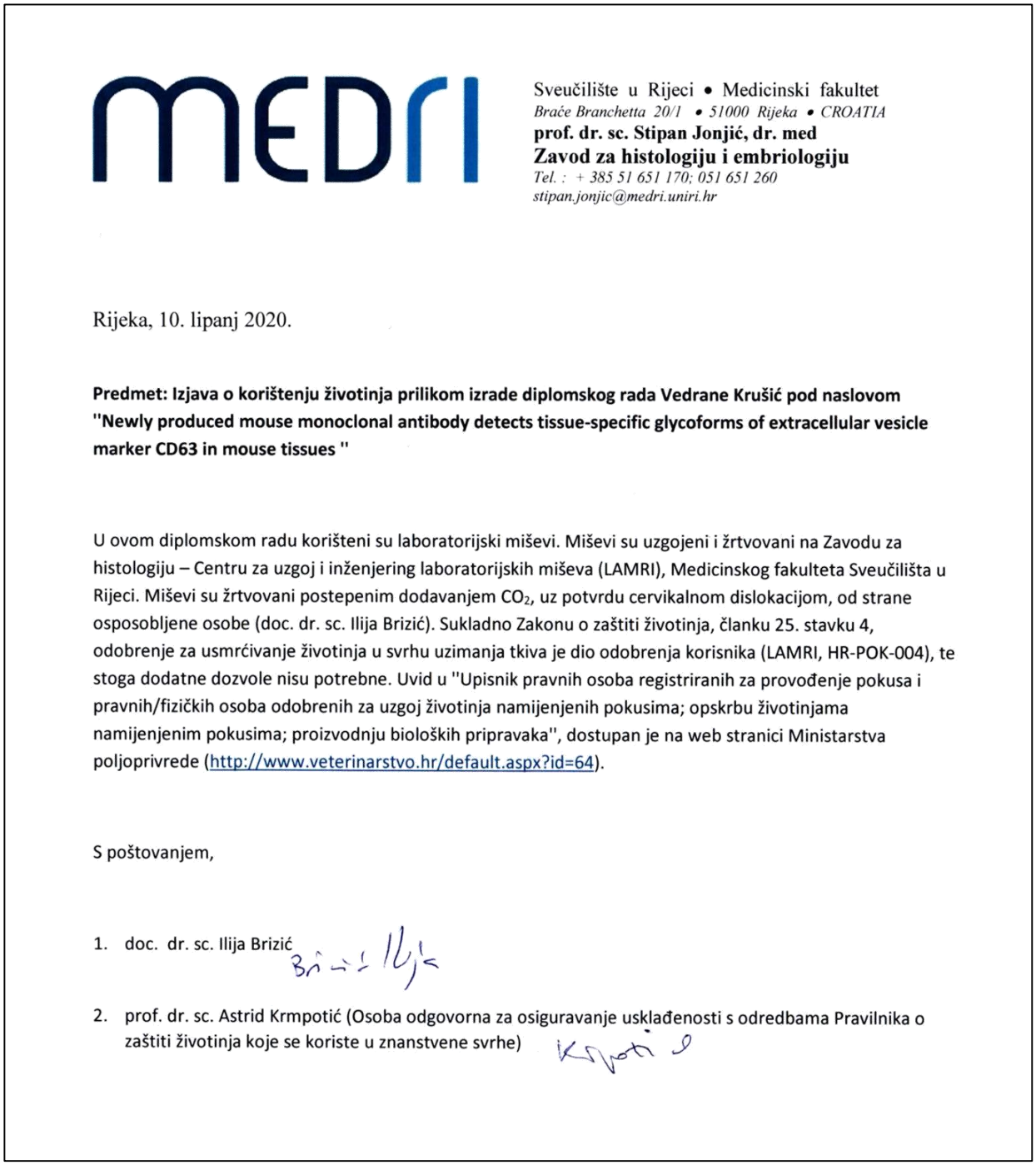
40. Sincock PM, Mayrhofer G, Ashman LK. Localization of the transmembrane 4 superfamily (TM4SF) member PETA-3 (CD151) in normal human tissues: comparison with CD9, CD63, and alpha5beta1 integrin. J Histochem Cytochem Off J Histochem Soc. 1997 Apr;45(4):515–25.

41. Schulze U, Brast S, Grabner A, Albiker C, Snieder B, Holle S, et al. Tetraspanin CD63 controls basolateral sorting of organic cation transporter 2 in renal proximal tubules. FASEB J Off Publ Fed Am Soc Exp Biol. 2017 Apr;31(4):1421–33.

42. Israels SJ, McMillan-Ward EM. CD63 modulates spreading and tyrosine phosphorylation of platelets on immobilized fibrinogen. Thromb Haemost. 2005 Feb;93(2):311–8.

8. Annex: Statement on the use of animals in research

In accordance with the Animal Protection Act, Article 25, paragraph 4, the authorization to sacrifice animals for the purpose of collecting tissue is part of the user's authorization (LAMRI, HR-POK-004) and therefore no additional permits are required. Below is copy of the corresponding statement issued by the LAMRI responsible persons.



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Rijeka, 10. lipanj 2020.

Predmet: Izjava o korištenju životinja prilikom izrade diplomskog rada Vedrane Krušić pod naslovom "Newly produced mouse monoclonal antibody detects tissue-specific glycoforms of extracellular vesicle marker CD63 in mouse tissues "

U ovom diplomskom radu korišteni su laboratorijski miševi. Miševi su uzgojeni i žrtvovani na Zavodu za histologiju – Centru za uzgoj i inženjering laboratorijskih miševa (LAMRI), Medicinskog fakulteta Sveučilišta u Rijeci. Miševi su žrtvovani postepenim dodavanjem CO_2, uz potvrdu cervikalnom dislokacijom, od strane osposobljene osobe (doc. dr. sc. Ilija Brizić). Sukladno Zakonu o zaštiti životinja, članku 25. stavku 4, odobrenje za usmrćivanje životinja u svrhu uzimanja tkiva je dio odobrenja korisnika (LAMRI, HR-POK-004), te stoga dodatne dozvole nisu potrebne. Uvid u "Upisnik pravnih osoba registriranih za provođenje pokusa i pravnih/fizičkih osoba odobrenih za uzgoj životinja namijenjenih pokusima; opskrbu životinjama namijenjenim pokusima; proizvodnju bioloških pripravaka", dostupan je na web stranici Ministarstva poljoprivrede (http://www.veterinarstvo.hr/default.aspx?id=64).

S poštovanjem,

1. doc. dr. sc. Ilija Brizić
2. prof. dr. sc. Astrid Krmpotić (Osoba odgovorna za osiguravanje usklađenosti s odredbama Pravilnika o zaštiti životinja koje se koriste u znanstvene svrhe)

9. Curriculum vitae

PERSONAL INFORMATION Krušić Vedrana

Braće Hlača 5, 51000 Rijeka (Croatia)

+385 917520409

vedrana.krusic@gmail.com

EDUCATION AND TRAINING

2018–Present **Master's degree programme: "Biotechnology in Medicine"**

Department of biotechnology, University of Rijeka, Rijeka (Croatia)

2015–2018 **Bachelor's degree in Biotechnology and Drug Research**

Department of biotechnology, University of Rijeka, Rijeka (Croatia)

2011–2015 **High school education**

"Gimnazija Andrije Mohorovičića Rijeka", science and mathematics programme

WORK EXPERIENCE

07/2019–Present **Master's thesis "Newly produced mouse monoclonal antibody detects tissue-specific glycoforms of extracellular vesicle marker CD63 in mouse tissues"**

- Faculty of Medicine, Rijeka (Croatia) – Department of physiology, immunology and pathophysiology
- Mentor: Assist. Prof. Kristina Grabušić, PhD

2018–2020 **Teaching assistant in Physical chemistry**

Department of biotechnology, University of Rijeka, Rijeka (Croatia)

07/2018 **Internship**

Jadran galenski laboratorij JGL d.d., Rijeka (Croatia)

2018 **Teaching assistant in Bioactive Components of the Mediterranean Diet**

Department of biotechnology, University of Rijeka, Rijeka (Croatia)

PERSONAL SKILLS

Mother tongue(s) Croatian

Foreign language(s)

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

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
Proficient user	Proficient user	Independent user	Independent user	Independent user

Digital skills - Self-assessment grid

Driving licence B

ADDITIONAL INFORMATION

Honours and awards

- The rector's award for academic excellence in the academic year 2019/2020
- Finished the Undergraduate university program "with highest honour" - summa cum laude
- University of Rijeka annual scholarship for excellence in academic years 2015/16, 2016/17, 2017/18, 2018/19 and 2019/20

Conferences

Krušić V, Brizić I, Shevchuk O, Miklić K, Lučin P, Jonjić S, Grabšić K. "In vivo study: Extracellular vesicle marker CD63 is differentially glycosylated in mouse tissues" International Student Congress Graz (2020). Abstract accepted for oral presentation, but conference cancelled due to COVID-19 outbreak.

Memberships Biotechnology *Student Association* at University of Rijeka (*USBRI*)