Chapter

# Engineering of Surface Proteins in Extracellular Vesicles for Tissue-Specific Targeting

*Stefan Vogt, Gerhard Stadlmayr, Johannes Grillari, Florian Rüker and Gordana Wozniak-Knopp*

## Abstract

Extracellular vesicles (EVs) have in the recent decades gained an important stand as vehicles enabling cell-to-cell transport and communication. With the advanced development towards their clinical use and increasing versatility of potential applications, improving their tissue-specific targeting in order to enhance their functionality in drug delivery opened as a challenging engineering field. In the past, the question of specific intercellular contact has been addressed by decoration of the EV surface with agents able of specific target recognition. An attractive possibility here is the modification of strongly overexpressed EV surface marker proteins towards recognition of target cells. As these proteins are involved in a plethora of biological functions in EV biogenesis, cargo targeting and intercellular transfer, a minimal impact on protein architecture upon modifications is desirable, which would also increase the stability of the exosomal preparation intended for therapeutic use. This chapter focuses on the possibilities of engineering of the EV marker proteins towards antigen-recognition units broadly applicable to endow EVs with tissue-targeting functionality.

**Keywords:** extracellular vesicles, exosomes, tetraspanin, tissue-specific targeting, exosomal drug delivery

## 1. Introduction

The transfer of extracellular vesicles (EVs) has emerged in the last two decades as a novel mechanism for intercellular communication. Nomenclature of EVs has by now been agreed to be based on biogenesis pathway. Therefore, EVs budding from plasma membranes are termed ectosomes or microvesicles, while exosomes are formed via the endosomal compartment within multivesicular bodies (MVB) which then are released by their fusion with the plasma membrane. Finally, when blebbing from apoptotic cells, the term apoptotic bodies has been retained [1]. Biomarkers to differentiate these EVs are still questionable, and in general, size is used to differentiate exosomes from ectosomes, while apoptotic bodies are considered to present phosphatidylserine on the outside and thus can be stained by annexin V. Exosomes (40–150 nm in diameter) are produced by formation of endosomal intraluminal vesicles (ILVs) in multivesicular bodies (MVBs) and are secreted by fusion of these vesicles with the plasma membrane [2, 3] (**Figure 1**).

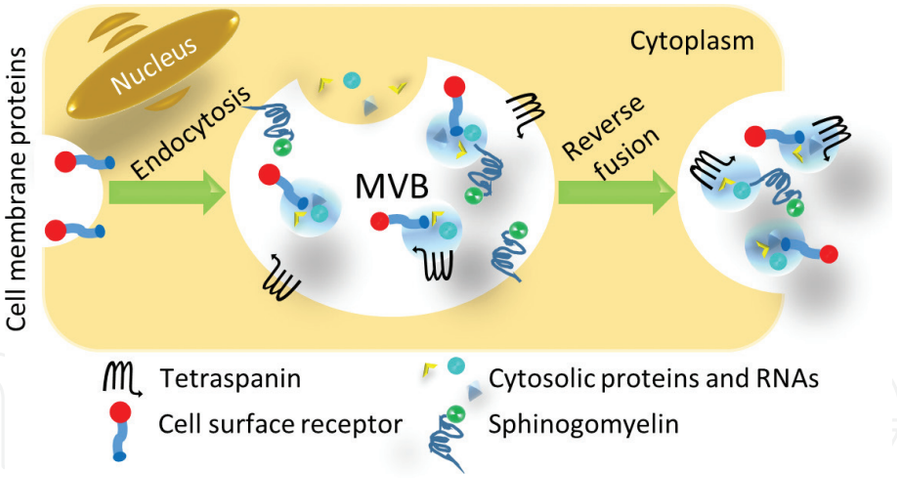


**Figure 1.**
*Biogenesis of EVs.*

Since they have been known to mediate directional intercellular transfer of their content, typically proteins, mRNAs, microRNAs (miRNAs) and a variety of non-coding RNAs [4], several studies were oriented towards their application as drugs *per se* or as therapeutic tool for drug delivery [5]. Their membrane is strongly enriched in sphingomyelin and cholesterol, which contributes to their unique buoyancy coefficient and enables practical isolation from other particles of cellular origin using differential centrifugation methods. Another discernible feature of exosome membranes is that it reflects the composition of the MVB membrane and has a high density of endosomal membrane proteins, such as proteins involved in MVB biogenesis (Alix and TSG101), membrane transport, in particular the components of the endosomal sorting complex required for transport (ESCRT) and most prominently tetraspanin proteins (CD9, CD37, CD53, CD63, CD81, CD82 and CD151) [6, 7]. Some tetraspanins such as CD9, CD81 and CD151 are more broadly expressed, while others are restricted to specific EV subsets [8, 9]. The overexpression of tetraspanins, as shown for CD9, can in several production cell lines enhance the exosome production and, in addition, cause a reduced overall size of the vesicles [10].

Tetraspanins play an important role in several physiological processes [11] and have been discovered to cooperate in states of health and disease in signal transduction, cellular activation, polarization, motility, adhesion, tissue differentiation, angiogenesis, tumorigenesis and metastasis [12–14], both by regulating cellular interactions as cell-membrane bound molecules and indirectly through exosomes. They are involved in each step of the metastatic cascade due to their ability to interact with cell surface receptors, adhesion molecules, matrix-remodeling proteases and signaling molecules. In this pathological state, they hence regulate cell proliferation, participate in epithelial-mesenchymal transition, modulate integrin-mediated cell adhesion and mediate the invasion through modulation of angiogenesis, tumor-endothelial cell interactions and regulation of cancer cell migration through the regulation of tumor microenvironment, as well as direct influence on extracellular matrix [15]. In this chapter, we introduce the characteristics of EVs and the engineering approaches aimed at their surface proteins to achieve tissue-specific targeting.

## 2. Structure of the tetraspanin proteins

The rod-shaped structure of a tetraspanin consists of four transmembrane helices that connect the two extracellular loops [16] (**Figure 2**). The short extracellular loop (SEL), which has not yet been reported to contain any element of a

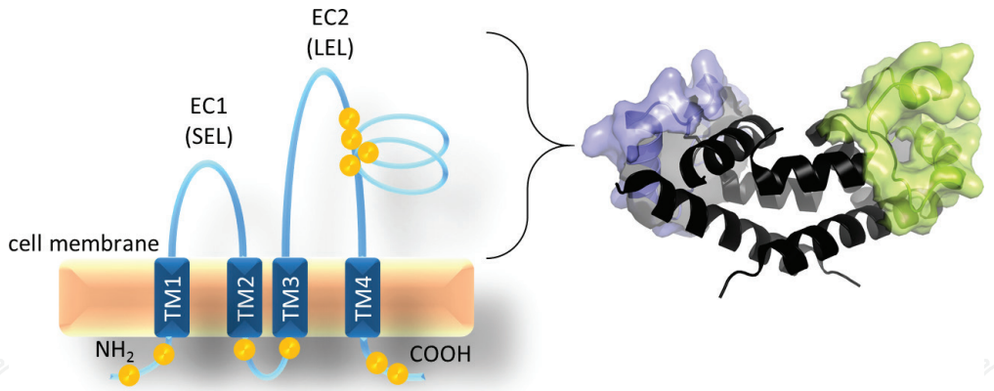


**Figure 2.**
*Organization of tetraspanin CD81: 4 transmembrane helices (TM) span 2 extracellular loops. Positions of cysteine residues are indicated with yellow dots. Crystal structure of EC2 domain is presented as a cartoon diagram of the dimeric form (PDB: 1G8Q ); the conserved three-helix bundle is black and the variable domains C and D are surfaced in blue or green for each protomer. The figure was prepared using PyMOL (PyMOL molecular graphics system, version 1.3 Schrödinger, LLC).*

particular secondary structure, is of 12–31 amino acids in length. In the studies oriented towards tetraspanin engineering, the EC1 domain has until now been less addressed due to its lower degree of structural organization. Its replacement for a strand of glycine-serine residues has led to an unusual cell surface expression and a distribution dissimilar to the wild-type parental molecule, which may suggest the importance of this structural subunit for the stability of the membrane-bound tetraspanin [17].

The large extracellular loop (LEL) or extracellular domain 2 (EC2) has on the other hand been well characterized. The crystal structure of CD81 [18] demonstrated the four invariant cysteine resides within EC2 to form the 2 disulfide bridges, as a hallmark stability feature of the conserved tetraspanin fold [8, 19] (**Figure 2**). Moreover, the topology of several other tetraspanins was predicted by molecular modeling studies in the absence of an available crystal structure, using the CD81 EC2 structure as a template: these were the tetraspanins CD37, CD53, CD82 and CD151 [20, 21]. It has been early established that binding of CD81-specific antibodies depends on the formation of the disulfide bonds [22]. One class of tetraspanins harbors the EC2 composed of five α-helical elements, forming stalk and head elements of a mushroom-like structure, and the second class of tetraspanins is postulated to contain an additional helical element, stabilized with another pair of cysteine residues. The EC2 fold structure is evolutionarily conserved across species for any particular tetraspanin in spite of a high degree of variability at the amino acid level [23], with a subdomain consisting of a three-helix-bundle fold and a second subdomain variable in size among members of the tetraspanin family [18, 20].

The presence of an energetically unfavorable hydrophobic patch and the dimeric form of CD81 LEL in a crystal hinted at the high likelihood of tetraspanin assembly into dimers or multimers in the cell membrane. As shown later, clustering of tetraspanins leads to the organization of tetraspanin-associated proteins in a tetraspanin web or tetraspanin-enriched microdomain (TEM) [24, 25]. Biologically, such domains induce partitioning of the complexes into lipid rafts and clustering of the lipid rafts [26].

Apart from the hydrophobic interactions of the extracellular domains, the membrane-proximal cysteines residing in transmembrane domains contribute to the dimer formation and stability of tetraspanins. For the CD9 tetraspanin, it has been demonstrated that these residues are positioned close to the dimerization interface and influence homotypic and heterotypic tetraspanin association depending on their reversible palmitoylation status [27]. Similarly, the mutation of N-terminal

and C-terminal transmembrane cysteines to serine eliminated palmitoylation of CD151, which turned out to be deleterious for the assembly with other cell surface proteins, including tetraspanins CD9 and CD63, their organization to TEMs and subsequently their subcellular distribution and cell morphology [28]. At the same time, it had minimal influence on the density of tetraspanin protein complexes and was dispensable for CD151-$\alpha_3\beta_1$ integrin association. Depalmitoylation of CD81 did not impact its surface expression and stability, but rendered it less available for contact with its natural interaction partner CD9 and the relevant epitopes less accessible for binding of structurally dependent antibodies [29].

The four transmembrane helices of tetraspanin proteins form two largely separated pairs of antiparallel helices: one pair comprises TM1/TM2 and the other TM3/TM4. The two pairs of helices only converge close to the cytoplasmic side of the membrane through contacts between TM2 and TM3. In the recently solved crystal structure of full-length CD81, this cone-like structure has been shown to harbor a binding pocket for cholesterol [30], and mutations within transmembrane domains in certain tetraspanins have been connected with pathological states [31]. The short cytoplasmic tails show no obvious functional significance in signaling processes, suggesting that their signaling competence relies on association with other molecules [32]; nevertheless, its mutation can lead to different assembly with association partners as shown for CD9 [33].

## 3. Natural ligands of tetraspanin proteins

### 3.1 The tetraspanin web and intertetraspanin contacts

The ability of members of the tetraspanin family to assemble into a unique biological feature known as tetraspanin-enriched microdomain (TEM) is due to their mutual interactions; however, these structures include also receptors, integrins and signaling molecules such as phosphatidyl-kinase C (PKC) and phosphatidylinositol-4-kinase (PI4K) [9]. These interactions are fundamental for cellular functions such as cell adhesion, proliferation and motility. Interactions between tetraspanin members are important in maintaining the integrity and stability of the tetraspanin web and providing binding sites for different ligands. The multimers of newly synthesized proteins are formed in the Golgi apparatus. The predominantly cross-linked tetraspanin species are homodimers, but also higher order complexes and low amounts of heterodimeric tetraspanins (CD81/CD9, CD9/CD151, CD81/CD151) were identified [27]. It has been suggested that tetraspanin homodimers, formed in the Golgi and present at the cell surface, serve as building blocks in the assembly of higher organized tetraspanin protein complexes. Interestingly, the exosomes originating from cell lines overexpressing CD9 are believed to be enriched in more stable TEMs [10]. Overall, although most tetraspanins can interact with most other tetraspanins, and similarly engage with several other proteins, the nature of these interactions has been until recently classified only according to their stability in the presence of detergents of different stringency, which does not necessarily reflect their significance in the cellular milieu [34]. A thorough characterization of strength and abundance of the interactions between the members participating in a tetraspanin web in a particular cell and physiological situation is therefore needed and will support the understanding of its mediated biological effects. Similarly, most data on tetraspanin functionality come from studies on their localization on cell membranes, while functional data in vesicles are still scarce. Therefore, we here summarize the known cellular functions, while speculating how this might translate to EVs.

An important step towards the understanding of the specificity of the tetraspanin interactions in TEM has been achieved by delineation of the involved tetraspanin regions by dissecting the model tetraspanin into domains, differently amenable for modification. Early experiments that addressed the relatively unstructured and at the same time antigen-binding competent regions that appeared attractive for mutagenesis resulted in a protein that showed aberrant clustering involving both homo- and heterodimerization of resulting full-length tetraspanins [35], albeit the mutagenesis method employed in this study was a complete deletion of targeted domains. The CD81 D-region was studied in more detail: the CD9 and CD151 tetraspanins were more competent of clustering with CD81 when homologously engrafted with CD81 D-region [36]. When the mutagenized CD81 EC2 molecular subunits were transplanted to other tetraspanins, the extremely flexible conformation of the solvent-exposed D-segment of CD81 EC2 was sufficient to overcome the orientational restrictions to initiate the homotypic contact for dimerization, and this finding has been corroborated with both wet-lab data and the insights from molecular dynamic simulation of the cell membrane-embedded protein [37].

### 3.2 Interaction of tetraspanins with integrins and matrix-degrading enzymes: role of tetraspanins in cancer and metastasis

Important association partners of tetraspanins are the integrins. The role of such complexes in invasive growth *in vivo* as well as the effect of integrin-mediated binding events on cell proliferation and invasion is well established. Especially, the laminin-binding integrins ($\alpha_6\beta_4$, $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_7\beta_1$) exhibit extensive interactions with tetraspanin proteins [12, 38]. The functionality of integrins may depend critically on their interaction of tetraspanins: it has early been described that the remarkably stable association of the tetraspanin CD151 and the integrin $\alpha_3\beta_1$ leads to a high level of activation of cellular PI4K [39]. Further, CD151 interacts directly with the $\alpha_3$ subunit and links it to other tetraspanins, CD9 and CD81. Loss of CD151 abrogates the $\alpha_3\beta_1$ mediated mobility on its ligands, laminin-332 and laminin-551. CD9/CD81 complex may even regulate the integrin-mediated functions independently of CD151 by forming a complex with the integrin and directing the PKC$\alpha$-$\alpha_3\beta_1$ association [40]. Another example of tetraspanin-integrin association reveals its proangiogenic role through VEGF induction, mediated by cooperation between TM4SF5 and integrin $\alpha_5$ of epithelial cells [41]. Interestingly, removal of CD151 palmitoylation sites did not disrupt the CD151–$\alpha_6\beta_4$ complex in epithelial cells but strongly influenced $\alpha_6\beta_4$-integrin–dependent cell morphology [42]. The rat tetraspanin D6.1A (human homolog is CO-029) was able to induce systemic angiogenesis by initiation of an angiogenic loop that reached organs distant from the tumor, probably due to the abundance of D6.1A in tumor-derived exosomes. This is in line with reports claiming that EVs prepare niches for metastatic tumor cells at tissues distant from the primary tumor [43]. This tetraspanin associates with integrins $\alpha_3\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$, as well as with tetraspanins CD9 and CD81, and is similarly to CD151 linked to tumor-promoting activities [44].

Active complexes of tetraspanins and integrins influence biological processes other than cellular signaling by interacting with cellular metalloproteinases, important players in the remodeling of extracellular matrix. A study of MDA-MB-231 cells, a breast cancer cell line, has indicated that the $\alpha_3\beta_1$-tetraspanin protein complex may be linked to an invasive phenotype of tumor cells via modulation of various signaling pathways, including activation of membrane metalloproteinase-2 (MMP-2), an enzyme associated with invasive migration of the cells, and affecting phosphatidylinositol-3-kinase (PI3K) signaling pathways, which control actin cytoskeleton dynamics [45]. By the incorporation of the members of a disintegrin and metalloproteinase (ADAM) family members the tetraspanins are able to influence the

cellular ectodomain cleavage and release activity of these enzymes [46]. The tetraspanins of the TspanC8 group (tetraspanins with 8 cysteines) have a significant impact on the cellular exit and catalytic activity of ADAM10 [47], in particular the activity of Tspan15/ADAM10 promoted N-cadherin cleavage [48–50]. Different TspanC8/ADAM10 complexes seem to have different substrate specificities [51]. The silencing of CD9 enhanced shedding of ADAM17-substrates TNF-α and ICAM-1 [52].

Important discovery of the possible consequences of fine differences in composition of TEMs has been delivered by the study of exosomes enriched in Tspan8-$\alpha_4$ complex that were preferentially taken up by the endothelial and pancreatic cells [53]. The fact that such modifications can allow selective targeting *in vitro* and *in vivo* holds promise to achieve improved exosomal delivery by engineering of their membrane components.

### 3.3 The role of tetraspanins in immune complexes

In antigen-presenting cells (APCs), tetraspanins integrate into TEMs protein-recognition receptors binding to conserved repeated motifs of microbes, such as Toll-like receptors, and MHCII molecules into tetraspanin web platforms, as well as $Fc_\gamma$ receptor I in phagocytic cells, $Fc_\gamma$ receptor IIb and III upon the activation of macrophages and $Fc_\varepsilon$ receptor I in monocytes and skin-derived dendritic cells [54].

The particular role of CD81 protein in the formation of specialized microdomains in the plasma membrane of the cells of the immune system was discovered by elucidating its function of recruiting various adhesion molecules, receptors and signaling proteins to the central zone of the immune synapse in T-lymphocytes and APCs [55]. Therefore, it has early been proposed for CD81 to play a key role during antigenic presentation, since it colocalizes with the T-cell receptor/CD3 [56], and CD81 indeed turned out to be a regulator of CD3 clustering and sustained CD3 signaling [57].

Further, the T-cell side of the immune synapse is densely populated by tetraspanins CD9 and CD151. The abolishment of their expression reduces markers of activation of T-lymphocytes conjugated to the APCs, such as IL-2 secretion and expression of CD69 [58].

Another role in the immune response of tetraspanin CD81 is amplifying and sustaining B-cell receptor signaling from lipid rafts by ligation to the co-receptor CD19/CD21 complex. The signaling through a variety of cell surface protein complexes implies a role of lipid rafts, again highlighting the ability of tetraspanin to facilitate raft association [9].

### 3.4 Tetraspanins in pathogen infection

Several studies have been oriented towards the research of tetraspanins as ligand molecules for pathogen entry. CD81 has been identified as a ligand for hepatitis C virus (HCV) recognition and viral entry [59]. The ligand for viral glycoprotein E2 is the D-domain of the LEL, a dynamic region positioned within the triple-bundle helix, whose conformation in solution differs substantially from the one suggested by crystal structure. Challenging for structure-based design, this region nevertheless presents an attractive target for design of therapeutically relevant ligands with methods such as NMR [60]. Apart of the EC2 domain, other regions of CD81 have proven important for virus infection. Experimental evidence here was based on the exchange of the structural domains of the molecule with the ones of tetraspanins of different degrees of homology, and it was found that closely related substitutions were more efficient at functional complementation of CD81. Viral entry has been shown to correlate with surface expression of the chimeric protein and to depend on the presence of the cholesterol-coordinating glutamate residue [61]. EWI-2wint,

a cleavage form of EWI-2, a member of the immunoglobulin superfamily, has an inhibitory effect on HCV infection by obstructing the interaction between CD81 and HCV E2 [62]. The related factor EWI-F inhibits *Plasmodium* infection, whereas its silencing increases infection efficiency [63].

Tetraspanin microdomains have been described to regulate HIV-1 entry, assembly and transfer between the cells [64, 65]. CD81 influences importantly the early stages of virus replication by controlling the stability of HIV-1 restriction factor and consequently the activity of viral reverse transcriptase [66], while CD63 facilitates endocytosis of the HIV receptor CXCR4 [67] as well as supports the replication steps in macrophages [68, 69]. Also Coronaviruses and low-pathogenicity Influenza A viruses utilize TEM domains as entry portals to co-engage with cellular receptors and proteases, which enable viral proteolytic priming [70]. As shown in an *in vivo* model with CD151 null-mice, this tetraspanin is a critical novel host factor of nuclear export signaling of Influenza A virus, used complementary to the viral nuclear export proteins [71].

Tspan9 modulates the early endosome compartment to make it more permissive for membrane fusion of early-penetrating viruses, and its depletion strongly inhibits infection by alphaviruses that fuse in early endosomes but does not alter the delivery of virus to early endosomes or change their pH or protease activity [72]. It is unclear, what function then EV-based tetraspanins might have in the context of viral infection, and it might be speculated that cells use EVs to titrate away virus into membrane structures that are unable to provide replication and protein synthesis machineries for the virus. This is supported by the findings that EVs might have anti-influenza infection activity *in vitro* [73].

## 4. Extracellular vesicles as mediators of cell-cell interaction

### 4.1 Biological basis for therapeutic applications: EVs as mediators of intercellular interactions

EVs are secreted by most cell types and are taken up by recipient cells, where their cargo consisting of a cocktail of proteins, mRNAs and non-coding RNAs alters the behavior of the recipient cells in a way that might be even considered similar to hormones or cytokines [74], e.g. in the context of skin or bone cell paracrine signaling [75–77]. siRNAs (small interfering RNAs) and miRNA-based inhibitors have been recognized as potent novel drug candidates for many years. As EVs can be loaded with different drugs *in vitro*, they qualify as an attractive drug delivery system. The specificity of the recipient cell targeting *in vivo* is understood in a limited way only, although there is evidence of accumulation of specific EVs [43]. For example, EVs from human mesenchymal stem cells accumulated in the liver, spleen and sites of acute kidney injury [78]. Such tropism for a specific cell type, a requirement for targeted drug delivery, appears to be determined by surface proteins of the source cells. The composition of EV membrane reflecting the one of their source cell makes these particles non-immunogenic, and their small size allows them to pass the immune surveillance of the host organism [79, 80]. The reported engagement of exosomes in physiological processes in normal and diseased central nervous system makes them attractive vehicles for delivering neurotherapeutic agents across the blood-brain barrier [81–83]. Nevertheless, their delivery in humans seems so far limited to liver and kidney as they are reported not to reach therapeutic amounts in brain, heart and other tissues due to lack of specific targeting and thus low enrichment of the intended therapeutic ingredient in the target tissue. Modifications of the EV surface membrane to achieve enhanced targeting of a specific cell type are hence a common strategy embodied in several different engineering approaches.

### 4.2 Mechanisms of EV entry into the target cell

When an EV attaches to the target cell surface, it can in some cases activate the cognate receptors without internalization or transfer of the content to the recipient cell via its fusion with the target cell membrane or via endocytosis [84]. Endocytosis is an active process that requires cytoskeletal remodeling dependent on actin dynamics and includes clathrin-dependent endocytosis, phagocytosis and macropinocytosis. The clathrin-dependent endocytosis has been established as cellular entry for EVs based on the experiments with specific inhibitors of this pathway. Additionally, endocytic uptake of EVs can involve lipid rafts, sometimes dependent on caveolin proteins. The size of EVs may be a limiting factor for cellular entry via endocytosis [85]. The EV uptake by phagocytosis was monitored by their high level of accumulation in phagocytic cells and localization into the phagolysosome [86], as well as the identification of the crucial role of the phosphatidylserine binding T-cell immunoglobulin and mucin domain containing (TIM4) receptor for the uptake of exosomes into macrophages [87, 88]. The contribution of macropinocytosis pathway was revealed with studies where exosomal uptake was decreased by the inhibition of cytoskeletal rearrangements that normally lead to membrane ruffles [89], as well as with its promotion caused by the activation of the agonistically acting epidermal growth factor [90].

## 5. Molecular engineering to facilitate EV labeling and delivery to target cells

### 5.1 Modifications of EV membrane with non-covalent and chemical modifications

The role of EVs as encapsulated intercellular messengers makes them attractive for development into nanoscale therapeutic agents [91], and therefore, the need of augmenting the interaction with the recipient cell has been widely recognized. The higher rigidity of the membrane of EVs in comparison with their source cells does not appear to obstruct the efficient application of common hydrophobic insertion strategies to EVs. The introduction of small lipophilic ligands, such as membrane dyes, works effectively for their labeling and aids in monitoring in *in vitro* and *in vivo* experiments. Furthermore, hydrophobic loading is used for encapsulation of certain drugs and leads to their increased stability and therapeutic effect [92, 93].

Due to the negative charge on the recipient cells, binding and uptake of EVs were enhanced by increasing the charge interactions with an artificially introduced positive surface potential, as exemplified in their derivatization with cationic lipids [94]. A downside of this method can be that the extremely charged cationic reagents can cause cytotoxicity and the cellular uptake of the modified particles can proceed differently from the usual pathways, which results in an unpredictable cellular fate of the particle and its cargo and possibly its undesired degradation. The innate slightly negative electrostatic potential exhibited by unmodified EVs should contribute to the longer half-life of such particles *in vivo* as inferred from liposomal studies [95]. Further, the extremes of unbalanced positive charge may negatively impact the storage stability and the application of such reagents in high concentrations.

As an alternative to other methods, labeling of EV surfaces with fluorescent probes has been achieved using click chemistry without an apparent effect on the size and function of the particles [96]. Nevertheless, there is an immense complexity behind the engineering and downstream methods developed for other

biologicals conjugated with small molecules, especially when intended for therapeutic purposes [97, 98].

The idea of decoration of EV membranes with functional ligands further led to the consideration of receptor binding strategies. Such EV functionalization can readily be achieved by modification of the source cell. A resulting opportunity harvesting the specificity of this approach could also be used as a strategy to eliminate vesicles implicated in pathological processes, such as cancer metastasis, or to neutralize the undesired activity of therapeutically applied vesicles. Transferrin-conjugated superparamagnetic nanoparticles, reactive with surface-expressed transferrin receptor of exosomes, enabled their isolation from blood and endowed the vesicles with superior targeting properties [99]. A robust labeling approach of microvesicles was the expression of biotin acceptor peptide-transmembrane domain (BAP-TM) receptor on the source cells in combination with biotinylation *in vivo* [100].

Another example of employing an EV-localizing protein for efficient presentation on the EV surface involved a peptide or a protein fused with the C1C2 domain of lactadherin, which binds to EV outer membrane due to its affinity to phosphatidylserine, strongly enriched in exosome membranes. This method was used to generate antibodies against tumor biomarkers [101] and to increase the host immune response to tumor-associated antigens [102, 103].

Strong binding of lactadherin to the exosome membrane was also the basis of an efficient labeling protocol utilizing overexpression of a fusion protein composed of *Gaussia* luciferase and a truncated lactadherin in source melanoma cells. After harvest by ultracentrifugation, the labeled exosomes were successfully used for *in vivo* biodistribution studies [104].

### 5.2 Engineering of tetraspanin proteins for detection and monitoring of EVs

Regarding the tetraspanins not only as very abundant, but as prominent structural and stability elements of the exosomal membrane, it is sensible to engineer genetic fusions of these proteins enriched in EVs to ensure a high density of the expressed product and optimize the derivatization for the different purposes of engineering: cognate ligand binding, labeling for visualization or isolation, modifying cargo uptake and transfer and stability (**Figure 3**). Most practically, transgene

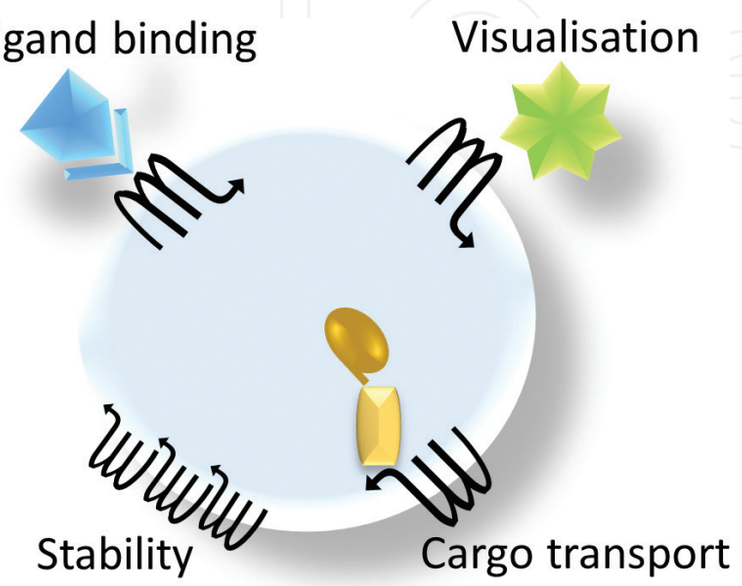


**Figure 3.**
*Main engineering goals utilizing the modification of tetraspanins.*

expression in the parent cell would be induced to deliver EVs enriched in the modified protein. The success of such set-up depends on the understanding of both molecular biology and protein architecture of the targeted species.

In one of the pioneering studies, certain sites on the tetraspanin CD63 have been chosen to allow the integration of fluorescent fusion proteins on the extra- and intravesicular side of the exosomal membrane [105]. CD63-GFP fusions have proven valuable reporters in the elucidation of the role of immune synapse in secretion of exosomes from T-cells to APCs and their fusion with recipient cells [106], in determining differential uptake properties of different immune cell subsets for EVs originating from a cancer cell line [107] and in an *in vivo* study imaging the fate of EVs produced by breast cancer cell line in *nude* mice [108].

### 5.3 Engineering of EV surface proteins for enhanced cell-type specific targeting: introduction of target specificity, stability and improved cargo delivery

In the pioneering example of derivatization of a protein enriched in exosomal membrane to achieve specific targeting [109], the rabies virus glycoprotein (RVG)-peptide fused with lysosome-associated membrane glycoprotein 2b (Lamp-2b) was used to target exosomes to the central nervous system in an *in vivo* mouse model. Immature dendritic cells-derived exosomes, enriched in an N-terminal fusion of an $\alpha_v$ integrin-specific internalizing RGD-sequence containing peptide with this scaffold, could internalize efficiently into target-positive breast cancer cells [110]. The display of such constructs could be efficiently enhanced by introducing a protective glycosylation motive, which improved the surface expression of exosome-bound N-terminally fused peptides by preventing their acid-mediated proteolysis during endosomal passage and indeed led to a more efficient specific cellular uptake of exosomes engineered in this way [111]. Specific targeting of IL3-receptor overexpressing chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells has been shown for exosomes armed with a fusion protein of IL3 and Lamp2b and loaded with Imatinib, a tyrosine kinase inhibitor, and led to a the reduction of tumor size *in vivo* [112]. Exosomes engineered in this way achieved a higher abundance at the tumor site and were hence able to inhibit xenograft growth more efficiently than the active ingredient alone or than loaded control exosomes.

Glycosylphosphatidylinositol (GPI)-mediated anchoring of the specific targeting unit was demonstrated to be a stable alternative to Lamp2b fusions. EVs expressing GPI-anchored nanobodies, specific to epidermal growth factor receptor (EGFR), displayed enhanced binding to the EGFR-overexpressing cancer cells. This, however, did not lead to an increased uptake, and it was suggested that in this particular biological system not only the affinity but also the density of the targeting ligand must be high enough to induce receptor clustering and subsequent internalization [113]. Exosomes derived from a HEK cell line transfected with a construct of platelet-derived growth factor (PDGF)-anchoring sequence and an EGFR-binding peptide were on the other hand efficient in targeting EGFR-expressing tumors *in vivo* and reducing their size with delivery of microRNA Let7 [114]. Enhanced uptake has been achieved for EVs enriched in a fusion protein of tetraspanin CD63 and stearylated octaarginine, a representative cell-penetrating peptide, by their ability to induce active macropinocytosis [115].

The concept of enhancing the stability of the targeted exosomal surface protein intended for fusion with a targeting agent to allow a higher degree of versatility for their modification for an improved target recognition has raised interest in their engineering at the protein level. A significant increase in thermal stability has been achieved by introduction of additional disulfide bonds in the EC2 of tetraspanin CD81, with the best variant exhibiting a positive shift in the melting temperature

for 45°C comparing with the wild-type protein [116]. When engrafted with a human transferrin-receptor specific peptide, the stabilized scaffold exhibited significantly better biophysical properties than the analogously engrafted wild-type protein. In the same study, a mutant has been discovered that exhibits reversible unfolding behavior up to a temperature of 110°C in contrast to the wild-type CD81 EC2, which presents another option for extensive engineering required for directed evolution of tetraspanin proteins towards novel antigen binding.

Not only can the overexpression of tetraspanins in source cell lines increase the production and stability of exosomes, but also the exosomes engineered to contain adeno-associated viruses (AAVs), designed for improved delivery of genetic material to target cell [100], were superior in their yield and functionality when CD9 was overexpressed in AAV-producer cells [10]. CD9 overexpression has also increased the speed and transduction efficiency of lentiviral gene delivery into numerous cell lines, confirming the important role of this tetraspanin in gene transfer [117].

The current scope of EV engineering reaches beyond receptor targeting systems and aspires towards modifications with complex modules, assigning them simultaneously with multiple novel functionalities, such as specific recognition as well as enzymatic activity, to enhance their potential therapeutic effect. Recently, regression of orthotopic Her2-positive tumors has been achieved by applying exosomes, able of specific targeting via their surface decoration with a fusion protein of high-affinity anti-Her2 single-chain Fv, and containing mRNA encoding a bacterial enzyme Hchr6, which in the strongly Her2-positive cells catalyzed the conversion of the prodrug CNOB to cytotoxic MCHB [118]. Tetraspanin engineering could support a sophisticated concept to aid intracellular delivery of exosomal cargo proteins, directionally incorporated during vesicle biogenesis [119]. Genes encoding two recombinant proteins, a fusion of photoreceptor cryptochrome 2 (CRY2) and the protein of interest and a fusion of tetraspanin protein CD9 and CRY-interacting basic-helix–loop–helix 1 (CIB1), were co-transfected into a single cell line. The blue light-induced binding of CRY2 and CIB1 enabled docking of CRY2-fused target proteins into nascent exosomes, and in the absence of the blue light, the cargo protein was released into the exosomal lumen. Transfer of Cre recombinase confirmed the efficiency of this system *in vitro* and *in vivo*.

## 6. Conclusions

The tetraspanins, well established as the biomarker proteins of extracellular vesicles, have been addressed for increasing EV stability and improving their function as delivery vehicles, both by assigning them with target recognition properties and modulating their cargo transfer. From the current point of view, the complexity of the tetraspanin-mediated interactions and signaling networks formed in a cell is yet to be discerned. Tetraspanins are known to interact naturally with a plethora of cell surface-bound ligands, which results in potent biological effects conveyed through different pathways; however, systematic evaluation of the affinity of the association with the interaction partners would assist in prediction of the consequential cellular processes as well as in determining optimal choice of the tetraspanin targeted for modification. There are recent reports describing modified tetraspanins mediating both surface protein interactions and an intracellular fusion-mediated enzymatic activity, which underline the feasibility of engineering versatile functions into tetraspanin proteins as fusion partners. The structural details on tetraspanins modified in this way, as well as the read-out revealing their actual behavior in the foreseen role and the influence of such modifications on

the fate of an EV preparation, will pave the way into the design and production of EV-based reagents as therapeutics.

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## Conflict of interest

The authors declare no conflict of interest.

## Author details

Stefan Vogt[1], Gerhard Stadlmayr[2], Johannes Grillari[3,4], Florian Rüker[1,2] and Gordana Wozniak-Knopp[1,2*]

1 acib GmbH (Austrian Centre of Industrial Biotechnology), Graz, Austria

2 Christian Doppler Laboratory for Innovative Immunotherapeutics, Department of Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria

3 Christian Doppler Laboratory for Biotechnology of Skin Aging, Department of Biotechnology, University of Natural Resources and Life Sciences (BOKU), Vienna, Austria

4 Evercyte GmbH, Wien, Austria

*Address all correspondence to: gordana.wozniak@boku.ac.at

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