Involvement of Tspan8 in exosome assembly and target cell selection

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“You can't cross the sea merely by standing and staring at the water.”

-Rabindranath Tagore (Indian Poet, 1861- 1941)

To my loving parents

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List of Abbreviations

a.a	Amino acid
AS	BSp73AS, Pancreatic carcinoma line
AP-2	complex of adaptins, involved in endocytosis
BMC	Bone Marrow cells
BSA	Bovine serum albumin
CLASPS	Cytoplasmic associated linker protein, involved in attachment of microtubules to the cell cortex.
CD	Cluster of differentiation
˚C	Degree Celcius
DHPE	Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt
DIC	Disseminated intravascular coagulation
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleotide
EC	Endothelial cell
EC1/2	Extra cellular loop 1/ 2
ECM	Extracellular matrix
ECL	Enhanced chemiluminiscence
EDTA	Ethyline Diamine tetraacetic acid
EE	Early endosome
ERM	Ezrin, Radixin, Moesin
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence-activated cell sorting
Fb	Rat Lung Fibroblast cells

FCS	Fetal Calf Serum
h	Hour(s)
IgG	Immunoglobulin G
ILV	Intra luminal vesicle
INS2	Intersectin-2
IP	Immunoprecipitation
i.v.	Intravenous
LN	Lymph node
LNStr	Lymph node stromal cells from
m	Milli
MHC	Major Histocompatibility Complex
µ	Micro
min	minutes
mRNA	Messenger RNA
miRNA	Micro RNA
MVBs	Micro Vesicular Bodies
nm	Nano meter
O/N	Overnight
PAGE	Poly acrylamide gel electrophoresis
PE	Phyco-erythrin
PEC	Peritoneal exudate cells
Φ	Amino acid with a bulky hydrophobic side chain
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween 20
PKC	Phospho kinase C
PMSF	Phenylmethylysulfonylfluoride

PS	Phosphatidylserine
Rab	Ras-related in brain
RAS	Proto-oncogene
RAEC	Rat aorta endothelial cells
Rho	Family of GTPases
RNA	Ribo nucleic acid
rpm	Revolutions per minute
RT	Room temperature
SC	Spleen cells
SDS	Sodium dodecyl sulphate
SH3	Src homology 3
siRNA	small interfering RNA
TEMED	Tetramethylethylenediamine
TEMs	Tetraspanin enriched microdomains
TM	Trans-membrane
V	Volts
WB	Western blot
Y	Tyrosine

Zusammenfassung

Exosomen gelten als wichtigste interzelluläre Kommunikatoren und Tetraspanine bzw. Tetraspaninkomplexe spielen hierbei eine große Rolle in der exosomalen Erkennung von Zielzellen. Es konnte gezeigt werden, dass Exosomen welche einen Tspan8-CD49d Komplex tragen bevorzug an Endothelzellen binden, wodurch Angiogenese ausgelöst wird. Dieser Befund war unerwartet, da in den Exosomen freisetzende Zellen Tspan8 mit CD49c und den Tetraspaninen CD9 sowie CD151 assoziiert ist. In Betracht des möglichen therapeutischen Nutzens von Exosomen als Botschafter und Medikamententransporter, ist es essentiell den genauen Mechanismus der Bildung des Tspan8-Netzwerkes in der Zellmembran gegenüber dem der Exosomen zu klären. Daher habe ich den Weg der Internalisierung von Tspan8 und Tspan8-Chimären, bei denen der N- und/oder C-Terminus gegen die korrespondierenden Regionen von CD9 oder CD151 ausgetauscht wurden, untersucht.

Nach Aktivierung der Zelle läuft die Tspan8-Internalisierung schneller ab als die CD9-Internalisierung und der Tspan8-CD9-CD151-Komplex in der Membran der ruhenden Zellen löst sich hierbei auf. Für die Tspan8-Internalisierung ist die Bindung seiner N-terminalen Region mit Intersectin-2, welches Teil eines multimodularen Komplexes, der in die Internalisierung von Clathrin-coated pits involviert ist, essentiell. Die Internalisierung und das Recycling von Tspan8 in frühen Endosomen wird durch die Rekrutierung von CD49d soweit verstärkt, dass lediglich in mit PMA aktivierten Zellen ein Tspan8-INS2-CD49d-Clathrin-Komplex in den cholesteroldepletionsresistenten Membranmicrodomänen gefunden werden kann. Die PMA-induzierte Tspan8-Internalisierung führt zu höherer Zellmigration, jedoch zu verminderter Matrix-Zell- und Zell-Zelladhäsion.

Unter der Annahme, dass dieser Mechanismuns der Tspan8-Internalisierung sowohl zum exosomalen Tspan8-Netzwerk, als auch zu den spezifischen Eigenschaften Tspan8-exprimiernder Exosomen beiträgt, führte ich eine komparative Analyse des exosomalen Tetraspanin-Netzes durch, um exosomale Zielzellen zu identifizieren.

Co-Immunopräzipitation von Zelllysaten und Exosomen aus den 4 Ratten-Tumorlinien AS, AS-Tspan8, AS-Tspan8/CD9n und AS-Tspan8/CD104, welche CD9, CD81, CD151

und die CD49c- und CD49d-Integrinketten exprimieren, sich aber in der Expression von Tspan8 und von der Integrinkette CD104 unterscheiden, zeigte sowohl im Zelllysat als auch im Exosomenlysat, dass CD9, CD81 und CD151 bevorzugt mit CD49c und nur CD151 ebenfalls mit CD104 assoziieren. Im Gegensatz hierzu assoziiert Tspan8 im Zelllysat mit CD49c, in den Exosomen jedoch mit CD49d und CD104. Die Unterschiede in der Zusammensetzung der Komplexe auf Exosomen beeinflusst die Zielzellspezifität in vitro und in vivo. In Vivo zeigte sich eine effiziente Aufnahme von Exosomen in verschiedene hämatopoetische Kompartimente und Organe nach i.v. Injektion in Ratten. Den Tspan8-CD49d Komplex exprimierende Exosomen integrierten bevorzugt in Endothelzellen und Pankreasgewebe, solche die den CD151-CD104 oder den Tspan8-CD104 Komplex trugen, wurden bevorzugt von stromalen Zellen der Leber und Lunge aufgenommen, und CD9- oder CD81-CD49c Komplexe exprimierende Exosomen wurden überwiegend in hämatopoetischen Zellen gefunden.

Zusammenfassend konnte ich den Weg der Tetraspanin8-Internalisierung aufklären und zeigen, dass sich veränderte Komplexzusammensetzungen während der Internalisierung bilden und in den Exosomen bestehen bleiben. Des Weiteren sind diese Unterschiede in den Tetraspanin-Komplexen für die Selektivität der Exosomen für bestimmte Zielzellen verantwortlich. Mit diesen Ergebnissen liegen zum ersten Mal sichere Hinweise auf die Rolle der exosomalen Tetraspanine in der Auswahl der Zielzelle vor, so dass die Zielzellen von Exosomen an Hand der Tetraspanin-Komplexe, die sie tragen, vorhergesagt werden können. Dieses Wissen stellt die Grundlage für die Generierung maßgeschneiderter Exosomen für den Transport von Therapeutische Mittel dar.

1. Introduction

Research so far has given us already a great insight into development and establishment of a tumor. Nonetheless, cancer still remains the second leading cause of deaths in the western world. Cancer mostly arises from one single cell which undergoes genetic malfunction leading to gain of function and/or of over representation of oncogenes or a loss of function of tumor suppressor genes. This endows the cancer cell with the capability of uncontrolled growth, evading the body's immune surveillance, and in some cases, metastasizing to new loci in the body.

Tremendous efforts in the field of tumor cell biology so far have equipped us with vast information about early tumor progression and thus give us ample understanding on tumor development, diagnosis and developing new treatment strategies. However, we still lag in the knowledge of late tumor progression which leads to metastasis which is the cause of death in 90% of cancer cases (Mehlen and Puisieux, 2006). A growing number of molecules have been already linked to this process, but there is still vast scope of understanding underlying mechanisms that enable the tumor cells to disseminate from the primary tumor site and settle into new, distant regions in the body forming metastasis.

1.1 Cancer: its development

Transformation from a normal cell into a tumor cell is a multistep process. To develop into a life threatening tumor, a cell has to acquire certain features which reflect genetic alterations that provide it with growth advantage and progressive transformation into cancer cell (Foulds, 1995). There are six hallmarks defined for metastatic cancer formation, namely, self-sufficiency in growth signals, insensitivity to growth-suppressive signals, evading programmed cell death, unlimited replicative potential, sustained angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2000). There are also some non-classical hallmarks of cancer cells which include: genetic instability, evasion of cell senescence, epigenetic alterations of cancer related-

genes, RNA interference alterations in the expression of cancer related-genes, changes in glucose and glutamine metabolism, participation of cancer stem cells in cellular proliferation, stromal cell participation in the tumor micro-environment, and changes in antigenic presentation and immunosuppression due to cytokines in the tumor micro-environment (Valdespino-Gomez et al., 2010). In this thesis, I would like to elaborate on sustained angiogenesis and tissue invasion and metastasis.

1.1.1 Sustained angiogenesis

All cells in a tissue lie within 100µm of a capillary for getting oxygen and nutrients. Formation of new tissue requires careful regulation of angiogenesis, the outgrowth of new blood vessels. To be able to progress into larger tumor mass, incipient neoplasias require developing angiogenic capacity (Bouck et al., 1996; Hanahan and Folkman, 1996; Folkman, 1997). Angiogenesis is also important for cancer cells to disseminate into peripheral blood and form micro metastases (Xie et al., 2009).

Positive and negative regulators of angiogenesis are carefully balanced and regulated. Vascular endothelial growth factor (VEGF) and acidic and basic Fibroblast growth factor (FGF1/2) are examples of angiogenesis-initiating signals which bind to cognate tyrosine kinase receptors on the surface of endothelial cells (Fedi et al., 1997; Veikkola and Alitalo, 1999; Otrock et al., 2008; Hicklin et al., 2005). Thrombospondin-1 and Pigment epithelium derived factor (PEDF) on the other hand, provide as inhibitors of angiogenesis (Bull et al., 1994; Chen et al, 2009). Cancer cells frequently outbalance the inhibitors to shift towards angiogenesis induction (Hanahan and Folkman, 1996; Vacca et al., 2000). Indeed, many tumors show upregulation of VEGF and FGF and impaired thrombospondin expression (Singh et al., 1995; Volpert et al., 1997). In addition, the ECM stores activators and inhibitors, which can be released by proteases that are expressed by tumor cells (Whitelock et al., 1996). Several studies have demonstrated the importance of sustained angiogenesis for tumor growth (Hanahan and Folkman 1996; Folkman 1997; Pan et al., 2007; Miao et al., 2000), and angiogenesis is a tempting target for therapeutic intervention.

1.1.2 Tissue invasion and metastasis

Malignant tumors are developed by invasiveness and metastasis, the settlement and growth of tumor cells at a distant site which is the cause of 90% of deaths of human cancers (Mehlen and Puisieux, 2006).

First step of metastasis is the dissemination of a tumor cell from the primary site. This requires epithelial to mesenchymal transition- like (EMT-like) phenomenon (Vernon and LaBonne, 2004; Savagner, 2010) when the expression of adhesion molecules goes down and a cell partakes a more migratory phenotype. The onset of EMT is associated with loss of cellular polarity, partial to total destabilization of cell–cell junctions, remodeling and replacement of cytoskeletal components, the onset of cell motility and the suppression of apoptosis (Moreno-Bueno, 2008).

Adhesion to the adjacent cells is lowered by modulation of functions of cell adhesion molecules (CAMs) of immunoglobulin, cadherin and integrin families. Most widely altered cell- cell interaction involves E-cadherin, which is ubiquitously expressed on epithelial cells. In normal cell, coupling via E-cadherin leads to anti-growth signal transmission via cytoplasmic contact with β-catenin to intracellular signaling circuits which include the Lef/ Tcf transcription factor (Christofori and Semb, 1999). E-cadherin function seems to be lost in majority of epithelial cancers, by mechanisms which include mutational inactivation of E-cadherin or β-catenin genes, transcriptional repression or proteolysis of the extracellular cadherin domain (Christofori and Semb, 1999). Changes in the expression levels of CAMs from immunoglobulin superfamily also play an important role in invasion and metastasis (Johnson, 1991), a clear example being N-CAM. In neuroblastoma, small cell lung cancer and Wilm's tumor, cells switch expression from a highly adhesive isoform of N-CAM to its poorly adhesive isoform (Johnson 1991; Kaiser et al, 1996), and there is shown to be an overall reduction in its expression in invasive pancreatic and colorectal cancers (Fogar et al., 1997).

Changes in integrin expression are also evident in invasive and metastatic cancers. By novel permutations of the α and β integrin subunits, carcinoma cells facilitate invasion by shifting their expression of integrins from the ones that favour the ECM in normal

epithelium to other integrins (e.g., α3β1 αVβ3 that preferentially bind the degraded stromal components produced by the extracellular proteases (Varner and Cheresh, 1996; Lukashev and Werb, 1998).

Another parameter of the invasive, metastatic capability are the proteases (Arribas et al., 2005; Trivedi et al., 2009). Cancer cells show upregulation of protease genes e.g. matrix metalloproteases (MMPs), downregulation of protease inhibitor (e.g., TIMP) genes and inactive zymogen forms are converted to active enzymes. In many cancers, the proteases are not released by the cancer cells themselves, but by the conscripted stromal and inflammatory cells (Werb, 1997; Rundhaug, 2003). After being released, these proteases may be wielded by the cancer cells. For example, several cancer cells induce urokinase (uPA) expression in cultured stroma cells, which binds to uPA receptor (uPAR) which is expressed on cancer cells (Johnsen et al., 1998). Another factor reported recently to be implicated in tumor migration and invasion are the microRNAs e.g. metastasis promoting miR-373 and miR-520c (Huang et al., 2008).

For transportation to secondary sites, cancer cells use the vascular or the lymphatic system. To enter the blood vessel, the cancer cell has to pass through the endothelium, a process called intravasation. Majority of the cells entering the blood stream die due to mechanical stress or immune surveillance (Jakobisiak et al., 2003). It is easier for cancer cells to enter the lymphatic system since the lymphatic endothelium has larger gaps (Alitalo and Carmeliet, 2002). On reaching the secondary site, the cancer cell is required to extravasate out of circulation. This means, the cell has to switch to adhesive properties again to be able to get fixed in the new settlement. It then has to adapt to the new environment to communicate with it, form cell-matrix and cell-cell contacts and acquire all the described capabilities again to form a massive tumor in the new site.

Working with a rat pancreatic tumor model, we became particularly interested in the question of tumor-initiated angiogenesis and metastasis.

1.2 The BSp73AS cell system

BSp73 is a spontaneously arisen rat pancreatic adenocarcinoma line. Passaging BSp73 cells subcutaneously several times revealed that the primary tumor obviously consisted

of two sublines/ clones. One of the clones, BSp73AS (AS) displayed very weak metastatic capability while the other, BSp73ASML (ASML) exhibited very strong metastatic potential (Matzku et al., 1983). Upon intra foot-pad injections in syngenic rats, the BSp73AS cells displayed large local tumors, but only reached the draining lymph nodes. In contrast to this, ASML cells showed very limited local tumor growth and rapidly spread through the lymphatic system to the lung and formed thousands of miliary metastases which were fatal for the animals.

Trying to define the differences between AS and ASML cells that account for the metastatic spread, molecules abundantly expressed in ASML cells, but absent in AS cells were identified by monoclonal antibodies and tested for their metastasis-promoting activity by cDNA transfection of AS cells. Thereby CD44v4-v7 was identified as a metastasis-promoting molecule. Instead, transfection with the cDNA of Tspa8 revealed a very surprising result. Rats bearing AS-Tspan8 tumors did not develop metastasis, but died due to DIC. This observation was particularly unexpected, as ASML cells grow miliary tumors due to their inability to induce angiogenesis. Thus, we followed, first that the tetraspanin Tspan8 is an angiogenesis inducer and second, that ASML cells likely express molecules that hamper Tspan8 initiated angiogenesis.

Before considering these questions, I briefly want to introduce the family of tetraspanins.

1.3 Tetraspanins

1.3.1 Structure and expression

Tetraspanins are small surface proteins that span the membrane four times and thus called tetraspanins. What distinguishes them from other proteins with four membrane-spanning domains is the presence of four hydrophobic, putative transmembrane domains (TM1-TM4), forming a small and a large extracellular loop (EC1 and EC2), with short intracellular amino and carboxyl tails. ECL2 can be subdivided into a constant region and a variable region. The constant region may account for dimerization, and the variable region for interactions with non tetraspanins partner molecules like integrins. Members of the tetraspanin family typically contain 4 to 6 conserved extracellular

cysteines linked with 2 to 3 disulfide bonds (Figure1). Four of these cysteines are absolutely conserved in all tetraspanins analyzed to date, including 2 cysteines present in a CCG motif, around 28–47 residues after the third transmembrane domain, and another cysteine about 11 amino acids before the fourth transmembrane domain. These conserved cysteines in the ECL2 are the hallmark of tetraspanins. Tetraspanins also typically contain conserved polar residues within transmembrane domains 1, 3, and 4, which stabilize the tertiary structure together with several other conserved residues (Boucheix & Rubinstein 2001, Berditchevski 2001, Todres et al. 2000, Hemler 2001, Stipp et al. 2003). The four transmembrane regions are responsible for intra and intermolecular interactions. They play an important role in the biogenesis and in tetraspanin-tetraspanin interactions to form the tetraspanin web or tetraspanin enriched transmembrane domains (TEMs). The intracellular juxtamembrane regions carry cysteine residues which are palmitoylated and are responsible for the tetraspanin-network formation (Yang et al., 2004). The cytoplasmic carboxy-terminus (c-terminus) interacts with cytoskeleton- proteins, signal transduction molecules, e.g., protein kinase C (PKC). In many tetraspanins for e.g., CD63, Net-1, CD-82, CD37, Tspan-3, CD151 the c-terminus contains a tyrosine based sorting motif: Tyr-Xa.a-Xa.a-Φ (YXXΦ), where represents any hydrophobic amino acid. This motif can bind to the adaptor proteins AP-1,-2 and -3 which are part of the Clathrin based sorting machinery (Stipp et al., 2003).

Most of the tetraspanins are glycosylated and this contributes to the variation in their molecular weight from 20- 50kDa (Yunta and Lazo, 2003). Most tetraspanins have N-glycosylation in the EC2 domain, with a few exceptions: CD9 is glycosylated in the EC1 (Boucheix et al., 1991) and CD81 and NET-2 are non-glycosylated (Oren et al., 1990).

The first characterization of a tetraspanin protein (ME491/CD63) at the sequence level appeared in 1988 (Hotta et al. 1988) and the existence of a family of related structures was first realized in 1990 (Wright et al. 1990, Oren et al. 1990). Tetraspanins are highly conserved proteins, some of them found in organisms as primitive as schistosomes and nematodes as well as in mammals.

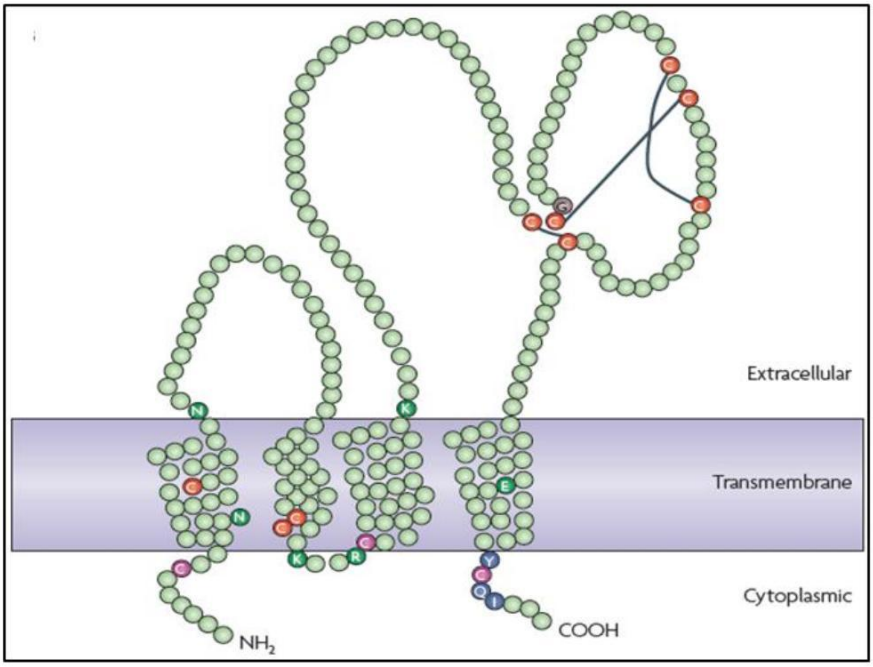


Figure 1. Structure of a prototype tetraspanin molecule, with amino terminal and carboxy-terminal intracellular domains. There is a large extracellular loop with 6 conserved cysteine residues (red) including the CCG motif, and disulphide bonds. The first and forth transmembrane regions have polar amino acids (green), the cytoplasmic domains contain palmitoylation sites (pink) and the carboxy-terminal domain contains a sorting motif (blue). *Adapted from Nature Reviews (Zoller, 2009)*

Certain tetraspanins have a restricted pattern of expression for example, CD53 is highly restricted to leukocytes (Maecker et al., 1997; Horejsi et al., 1991); CD37 is present exclusively on B-cells (Schwartz-Albiez et al., 1988); uroplakin on bladder epithelium (Walz et al., 1995) and RDS/Peripherin is expressed in the retina (Travis et al., 1991). Others, such as the leukocyte differentiation antigens CD81 and CD82, which were originally described on hematopoietic cells, can be found on most cultured cells. All mammalian cells express some members of the tetraspanin family, with the exception of red blood cells, which do not express any (Boucheix et al., 2001).

1.3.2 The Tetraspanin web

Individual tetraspanins can form complexes with a large number of membrane and cytosolic proteins that are required for their functions (Hemler, 2005; Andre et al., 2006; Levy and Shoham, 2005; Zoller, 2009). Their most prominent non-tetraspanin partners are integrins e.g., α3β1, α4β1 and α6β1. Tetraspanins are also shown to associate with growth factor receptors (Murayama et al., 2008; Sridhar et al., 2006), G protein coupled receptors (GPCRs) and their associated intracellular heterodimeric G-proteins (Little et al., 2004), several membrane proteases (ADAM10, TADG-15, and CD26/ dipeptidyl

peptidase IV), transmembrane proteins associated with tumor metastasis like CD44 and Epithelial cell adhesion molecule (EPCAM) and members of immunoglobulin superfamily like EWI-F and EWI-2. Some signal transduction molecules found to associate with tetraspanins are Protein kinase C (PKC), type II phosphatidylinositol 4-kinase (PI4KII) and phospholipase Cγ (PLCγ) (Hemler, 2005; Andre et al., 2006; Zhang et al., 2001; Claas et al., 2001).

Tetraspanin interactions can be classified as type I, II and III interactions depending on the strength of detergent required to break these associations, type I interaction being the strongest. The type I, direct protein-protein interactions are rare. These include tetraspanin homodimers, homotrimers and homotetramers, and heteromeric interactions between CD151 and some integrins, and between CD9, CD81, Tetraspanin8 and EWI proteins. Such direct interactions take place through the EC2 or the 2nd, 3rd or 4th TM region in the tetraspanin molecules. Most of the tetraspanin-integrin and the tetraspanin-tetraspanin interactions are type II interactions, and depend on the palmitoylation of tetraspanins and possibly the partner proteins. . Such interactions probably initiate in the golgi and provide target sequences for the co-associations that may follow (Berditchevski et al., 2002; Charrin et al., 2002; Zhou et al., 2004, Zoller, 2009). Type III interactions as with kinases, are also stabilized by palmitoylation (Hemler, 2005; Levy and Shoham, 2005; Berditchevski, 2002; Charrin, 2002).

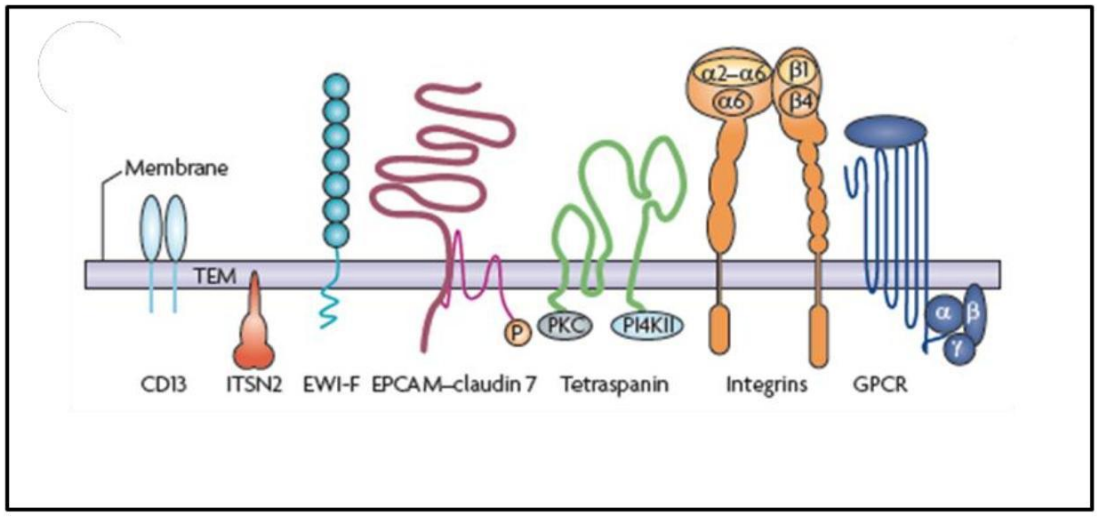


Figure 2. The tetraspanin web. The molecules: EWI-F, CD13 and Intersectin2 (ITSN2), integrins, EpCAM and GPCRs as part of the Tspan8 web. *Modified from Nature Reviews (Zoller, 2009).*

Besides these primary interactions, tetraspanins also associate with cholesterol and gangliosides (Charrin, 2003; Odintsova, 2006) which enable a higher order tetraspanin-complex formation resulting in microdomains called tetraspanin enriched membrane micro-domains (TEMs) and provide a signaling platform (Hemler, 2003; Hemler, 2005).

1.3.3 Functions

Owing to the dynamic nature of the tetraspanin microdomains (Devaux, 2004) and the reversibility of the palmitoylation status (Bijlmakers and Marsh, 2003; Linder et al., 2007) of this molecule, tetraspanins are involved in a multitude of biological processes. What adds to this divergence is that tetraspanins can either act directly, or as molecular facilitators, bringing the actual players in close vicinity.

Tetraspanins can promote spreading, migration and cable formation on extracellular matrices. Tetraspanins carry out these activities by compartmentalization of integrins, integrin internalization and recycling, or modulating integrin signaling (Hemler, 2005; Stipp et al., 2003; Berditchevski, 2001; Levy and Shoham, 2005, Zoller, 2009).

Tetraspanins are also supposed to be important in cell adhesion by regulating trafficking and biosynthesis of associating integrins e.g. CD151 affects cell adhesion and migration on Laminin 5 via integrin α3β1 and α6β4 recycling (Winterwood et al., 2006). It has also been shown that loss of CD151 upregulates RhoA activation, loss of actin organization at cell-cell junctions and increased actin stress fibres at the basal cell surface. This implies that CD151 is also an important regulator of stability of tumor cell-cell interactions, potentially through its interaction with integrin α3β1 (Johnson et al., 2009). CD151 is also known to regulate tumorigenesis by modulating the communication between tumor cells and endothelium, depending on its association with integrin α3β1 and α6β4.

Tetraspanins can also modulate cell-migration by their association with or recruitment of EWI-1 and-2 proteins which interact with ezrin–radixin–moesin proteins (ERM proteins) and down-regulate their phosphorylation. Phosphorylated ERM proteins can link the actin cytoskeleton with transmembrane proteins (Sala-Valdes et al., 2006) and thus affect cell migration. Tetraspanins also play a role in protein trafficking for e.g.,

CD63 has been shown to serve as an adaptor protein that links its interaction partners to the endocytic machinery (Duffield et al., 2003) association of CD151 with AP-2 which participate in endocytosis via the Clathrin coated vesicles (Stipp et al., 2003).

Several Tspans such as CD9, CD81 and CD82 regulate the activity of ADAM10 towards several substrates and illustrate how membrane compartmentalization by Tspans can control the function of cell surface proteases such as ectoproteases (Arduise et al., 2008). Tspans CD81 and CD82 affect myeloma cell fate via Akt signaling and FoxO activation. This anti myeloma effect of CD81/CD82 involves a down regulation of Akt, activation of FoxO transcription factors and decrease in mTOR and mTOR/ rictor (Lishner et al., 2008).

There are in addition, strong evidences that tetraspanins are also involved in fusion process, synaptic contacts at neuromuscular junctions, platelet aggregation, maintenance of skin integrity, immune response induction. For example, CD9 knock-out mice were infertile (Miyado et al., 2000) since their CD9 null eggs were incapable of fusing with sperms. Tetraspanins also have various roles in the life cycle and entry of different viruses like human T-cell leukemia virus1 and HCV type 16 (Spoden et al., 2008) in the host cell. CD63 was found to be associated with sites of HIV-1 assembly and can be found later in the viral membranes (Garcia et al., 2005). Another tetraspanin, CD81 has been implicated in the entry of HCV into its natural host cells (Cocquerel et al., 2006).

1.4 Tetraspanin 8 (Tspan8)

Tetraspanin 8 (D6.1A in rat and CO-029 in humans) originally was shown to be overexpressed in colorectal cancer (Sela et al., 1989) and described as a tumor-associated antigen in several human cancers (Szala et al., 1990) like colorectal, pancreatic and hepatocellular carcinoma (Zoller et al., 2006).

Tspan8 interacts with other tetraspanins namely, CD9, CD81, CD151 and several integrins, including α3β1, α6β1, α4β1. Tspan8 also associates with non-integrin partners including EWI-F, EpCAM, CD13 (Claas *et al.*, unpublished observations), PKC and PI4KII, EpCAM, CD44v4-v7 and α6β4 (Claas et al., 2005; Claas et al., 1998; Herlevsen, 2003; Gesierich, 2005).

1.4.1 Tetraspanin 8 and tumor progression

Profiling the tetraspanin web in human colon cancer cells revealed that Tspan8 was expressed in two metastatic sub-lines but not in a non-metastatic sub-line (Le Naour et al., 2006). High Tspan8 expression in a metastasized cell line SW620 compared to the primary tumor-derived colon carcinoma line SW480 from the same patient (Huerta et al., 2003) supports a role of Tspan8 in tumor progression. Tspan8 overexpression correlates with poor differentiation and intrahepatic metastatic spread of hepatoma, with only a hepatoma clone over-expressing Tspan8 developing intrahepatic metastases (Kanetaka et al., 2003).

The connection between Tspan8 and metastasis has been suggested to rely on its motility and survival supporting activities. There is strong evidence that Tspan8 promotes motility mostly through its association with α6β4. Although not constitutively, Tspan8 associates with α6β4 after hemidesmosome disassembly, which is accompanied by transient internalization of α6β4–CD151 and Tspan8 complexes, changes in cell shape towards a migratory phenotype, increased motility and hepatic metastasis formation (Huerta et al., 2003; Herlevsen et al., 2003). It is noteworthy that certain integrins are continually internalized from the plasma membrane to the endosomal compartment and are recycled back to the cell surface within 30 minutes through short-loop recycling under the control of RAB4A or through a long recycling loop through the perinuclear compartment under the guidance of RAB11A129 (Caswell and Norman, 2006).

Tspan8 has also been shown to promote migration, invasion and metastasis in oesophageal cancer by inducing upregulation of ADAM10 expression, which is a membrane anchored disintegrin metalloprotease (Zhou et al., 2004). It has also been observed that high expression levels of Tspan8 are associated with increased resistance to apoptosis (Huerta et al., 2003; Kuhn et al., 2007), where Tspan8 associated PKC probably plays important role (Kuhn et al., 2007; Ladwein et al., 2005).

1.4.2 Tspan8 and angiogenesis

Early works in our lab delivered first hints towards a metastasis-independent activity of Tspan8. In line with the metastasis promoting activity of Tspan8, AS cells transfected with Tspan8 and β4 showed increased metastatic potential (Herlevsen, 2003). Instead, as already mentioned, when AS line transfected with Tspan8 was injected into rats, lethally disseminated intravascular coagulation (DIC) developed which could be prevented by administration of the Tspan8 specific antibody (Claas et al., 1998). This suggested Tspan8 engagement in angiogenesis. DIC is a prothrombic state which is often seen in cancer patients, and tumor- associated angiogenesis and leakiness of tumor vessels are considered most important for this process (Zoller, 2009).

Subsequently, it was shown that DIC is a sequel of systemic angiogenesis induction via Tspan8 expressing exosomes (Gesierich et al., 2006). It was shown in our lab that Tspan8-overexpressing tumor cells induce angiogenesis in vivo, and tumor cells as well as exosomes derived thereof profoundly increased endothelial cell branching in vitro. Tumor cell-derived Tspan8 stimulates angiogenic factor transcription, which includes increased matrix metalloproteinase (MMP13) and urokinase-type plasminogen activator secretion, pronounced vascular endothelial growth factor expression in fibroblasts, vascular endothelial growth factor receptor (VEGFR) expression, and strong Tspan8 up-regulation in sprouting endothelium. Thus, Tspan8 initiates an angiogenic loop that, probably due to the abundance of Tspan8 in tumor-derived exosomes, reaches organs distant from the tumor. The Tspan8-specific antibody blocks angiogenesis effectively and with high selectivity for sprouting endothelium, where Tspan8 is highly upregulated (Gesierich et al., 2006).

As already mentioned, the integrin profile of the tumor cell is decisive of whether Tspan would induce tumor cell migration/metastasis or angiogenesis. Angiogenesis induction dominates in the absence of α6β4, which may actively suppress this process (Gesierich, 2006). Given that angiogenesis in this model is initiated by tumor cell-derived exosomes, the question arose, how Tspan8 may become integrated in exosomes.

1.5 Exosomes

Exosomes are 40–100 nm diameter membranous vesicles of endocytic origin that are released by a variety of cell types into the extracellular space (Simpson et al., 2008). Inward budding of endosomal membranes results in the progressive accumulation of intraluminal vesicles (ILVs) within large multivesicular bodies (MVBs). Transmembrane proteins are incorporated into the invaginating membrane while the cytosolic components are engulfed within the ILVs (Van Niel, 2006). Based on their biochemical properties, intracellular MVBs can either traffic to lysosomes where they are subjected to proteosomal degradation i.e., 'degradative MVBs' or, alternatively, to the plasma membrane (PM) where upon fusion with the PM they release their contents (ILVs) into the extracellular space, the so-called 'exocytic MVBs'. ILVs released into the extracellular space are referred to as 'exosomes'. To date, exosomes are the only type of membranous vesicles originating from intracellular compartments such as the MVBs. Endosomal Sorting Complexes Required for Transport (ESCRTs), multi-protein complexes, are involved in the mechanism governing the biogenesis/degradation of MVBs (Babst, 2005; Babst, 2006) in a ubiquitinylation-dependent (Hurley, 2008) manner.

Certain mechanisms for protein sorting to MVBs have been elucidated and include ubiquitination of the target and preferential aggregation. As shown in Figure2, Clathrin coated pits (include ubiquitinated proteins and proteins with other sorting signals that bind to AP-2 or Clathrin associated sorting proteins (CLASPs) (Traub et al., 2007)), aggregation of proteins in lipid rafts and TEMs can act as sorting signals to MVBs. A key player in MVB biogenesis is the hetero-oligomeric protein complex, endosomal sorting complex required for transport (ESCRT). ESCRT-I, -II and -III recognize mono-ubiquitinated cargoes and promote their inclusion in MVBs (Piper et al., 2007). Once completed, the ESCRT complex dissociates from the MVB membrane aided by the adenosine triphosphatase vacuolar protein sorting 4 (Vps4) and is recycled for subsequent cargo. However, some proteins such as the transferrin receptor are present in ILVs but are not ubiquitinated. Some studies also indicate that the transferrin receptor can interact with the ESCRT machinery despite the lack of ubiquitination (Geminard et al., 2004). These proteins, which lack the sorting signal for ubiquitination, are

partitioned into the ILVs based on their intrinsic physical properties and preference to segregate into raft-like microdomains (de Gassart et al., 2003). Protein clustering appears to be a major determinant in protein trafficking to the MVB.

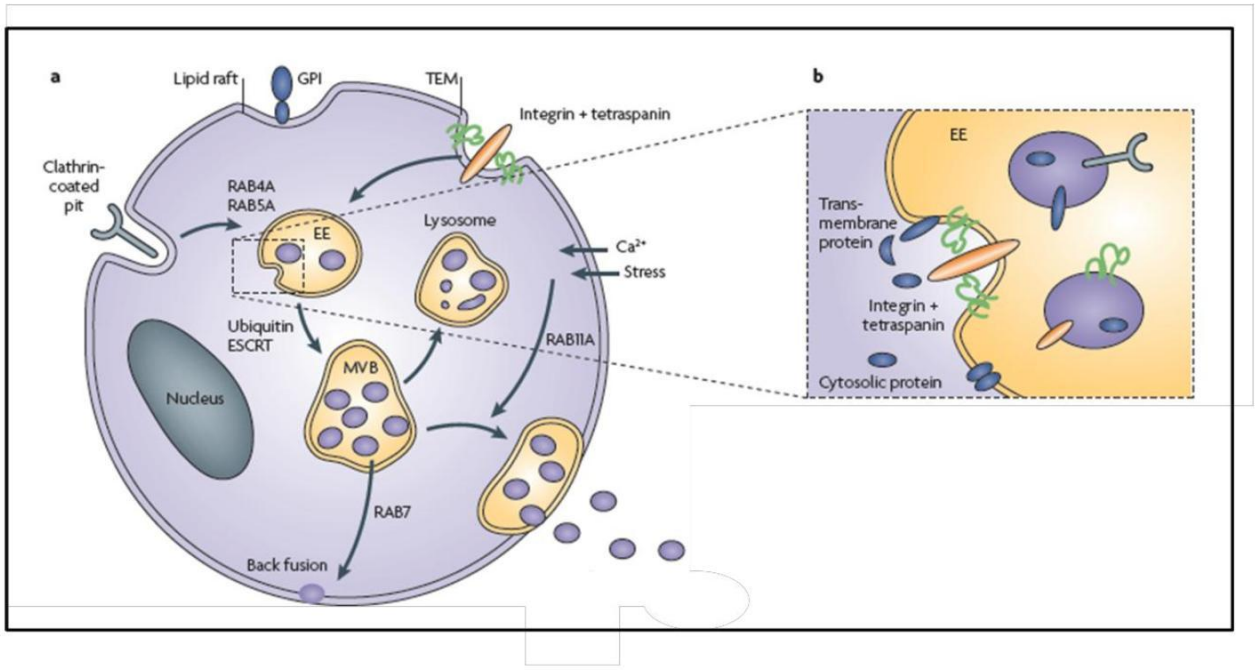


Figure 3. The generation of exosomes: a, b: Clathrin-coated pits, clustering of proteins in lipid rafts or tetraspanin-enriched membrane microdomains (TEMs) act as sorting signals, where endocytosed membrane proteins follow several routes from early endosomes (EEs) to recycling endosomes or multivesicular bodies (MVBs). MVBs derive from membrane invagination of EEs, the inward budding vesicles being defined as intraluminal vesicles. Ubiquitylation, Rab proteins and the endosomal sorting complexes required for transport (ESCRT) machinery, among others, are involved in the intracellular transport of the vesicles and help to sort cargo into the MVBs. The intraluminal vesicles either fuse with lysosomes for degradation, are released in the cytoplasm in a process called back fusion or are delivered as exosomes. *Modified from Nature reviews (Zoller, 2009).*

Exosome composition varies with the cell of origin. Nonetheless, exosomes contain a number of common protein components (Thery et al., 2001). The cytosolic proteins present on exosomes include Rabs, which promote exosome docking and the membrane fusion events (Mears et al., 2004). The annexins, including annexin I, II, V and VI, may regulate membrane cytoskeleton dynamics and membrane fusion events (Futter et al., 2007). Several adhesion molecules such as intercellular adhesion molecule-1, CD146, CD9, milk-fat-globule EGF-factor VIII (MFG-E8), CD18, CD11a, CD11b, CD11c, CD166 and LFA- 3/CD58 have also been identified in exosomal preparations (Thery et al., 2001; Mears et al., 2004). In addition, several proteins involved in apoptosis e.g., thioredoxin peroxidase II, Alix, 14-3-3 and galectin 3, as well as heatshock proteins Hsp70 and Hsp90, which can facilitate peptide loading onto major histocompatibility

complex (MHC) I and MHCII (Gastpar et al., 2005) are present on exosomes. As mentioned, one of the characteristic features of exosomes is the tetraspanins, which include CD9, CD63, CD81 and CD82. Exosomes also carry some cell-specific proteins like MHCII and CD86 present only on exosomes isolated from antigen-presenting cells (APCs) (Segura et al., 2005) and MFG-E8/ lactadherin present on exosomes from immature DCs (Veron et al., 2005). They are also enriched in proteins that participate in vesicle formation and trafficking like the lysobisphosphatidic acid (LBPA)-binding protein Alix (Futter et al., 2007). Other proteins like the metabolic enzymes such as peroxidases, pyruvate and lipid kinases and enolase-1 (Hegmans et al., 2004) are also detected on exosomes. Consistent with their endosomal origin, exosomes typically do not contain endoplasmic reticulum, mitochondria or nuclear proteins.

1.6 Tetraspanins and Exosomes

Exosomes have a wide variety of functions such as secretion of some proteins like transferrin receptor from red blood cells during maturation; in ectodomain shedding and consequently a vehicle for the cellular export of soluble molecules like L1 (CD171) and CD44 (Stoeck et al., 2006). Exosomes also play a role in antigen presentation (Wolfers et al., 2001) and are also implicated in immune suppression (Peche et al., 2003; Kim et al., 2005). Besides other functions exosomes are abundantly released by tumor cells (van Niel, 2006; Fevrier and Raposo, 2004; de Gassart et al., 2004; Lakkaraju et al., 2008). Exosomes are deemed to be indispensible for intercellular communication by the transfer of proteins, mRNA and miRNA in targeted cells, which can have severe consequences for the target cell by initiating activation of signaling cascades, inducing gene transcription, or RNA silencing (Février and Raposo, 2004; Schorey et al., 2008; Simpson et al., 2009; van Niel, 2006). Notably, it is known for long that tetraspanin complexes are constitutive components of exosomes (Escola et al., 1998; Hemler, 2003; Zoller, 2006). However, whether tetraspanins are functionally relevant components of exosomes remained elusive for a long time. We have recently attacked this question in the above described model of Tspan8-exosome-induced angiogenesis. First to note, we could confirm the striking power of exosomes in intercellular communication (Gesierich et al., 2006). AS-Tspan8-derived exosomes significantly altered protein expression and RNA transcription in endothelial cells and sufficed not only for EC activation, but also

for EC progenitor maturation. Furthermore, the effects of AS-Tspan8-derived exosomes on endothelial cells could be inhibited by D6.1 and anti-CD49d, but not by anti-CD151, anti-CD9 or anti-CD49c (Nazarenko et al., 2010). These latter findings were somewhat surprising as AS-Tsan8 cells express CD9 and CD151 at high levels and Tspan8, CD9 and CD151 preferentially interact with CD49c rather than CD49d.

1.7 Aim of the thesis

As described and expected, AS-Tspan8-derived exosomes mostly affected endothelial cells. Though expected, the molecular basis was not at all clear. This also accounted for the inefficacy of CD151 and CD9 in angiogenesis induction as well as for the contribution of CD49d and the apparent failure of involvement of CD49c. To shed light on these open questions, I started to search for peculiarities of Tspan8 recruitment into exosomes where the first step will be the internalization and endosomal recovery.

Tetraspanins can become internalized via a tyrosine-based internalization motif, YxxΦ. It is possible that this position close to the membrane disrupts the binding of the μ subunit of the AP-2 adaptor complex (Berditchevski, 2007; Nakatsu et al., 2003, Zoller, 2009). The motif not being present in all tetraspanins (CD9) or being located too close to the plasma membrane (Tspan8) (Berditchevski, 2007), tetraspanins may alternatively become internalized via associated molecules with an YxxΦ motif (Aridor et al., 2002; Xu et al., 2009). CD151 and CD49d both contain a tyrosine-based internalization motif (Berditchevski et al, 2007; Bonifacino and Traub, 2003; Pandey et al., 2009). Thus, CD9 and/or Tspan8 could become internalized via their association with either of these molecules. However, because only AS-Tspan8-, but not AS-derived exosomes interact with EC (Gesierich et al., 2005; Nazarenko et al., 2010), a common path of Tspan8 and CD9 internalization via CD151 and CD49d became unlikely.

To answer the question, how Tspan8 becomes recruited into exosomes, we sought to define the region(s) of Tspan8, which are important for the obviously quite selective internalization of Tspan8. To foray into this research, I exchanged the N- and/or the C-terminal regions of Tspan8 by the corresponding regions of CD9 and CD151 or, the large extracellular loop of CD9 and CD151 was exchanged with that of Tspan8.

Subsequently, I searched for molecules associating with the internalization-relevant Tspan8 regions. This included the exploring how CD49d may come into play.

On the basis of these results and having unraveled the Tspan8 internalization complex, I started to explore the engagement of this internalization and exosomal Tspan8 complex in target cell selection.

2. Material and Methods

2.1 Material

2.1.1 Instruments

Name	Company
Agitator for bacterial cultures	Edmund Buehler GmbH, Hechingen
Camera system Spot CCD	Diagnostic Instruments, Sterling Heights, USA
Cell chamber Neubauer improved	Brand, Wertheim
Centrifuge Sorvall RC5B Plus	Kendro, USA
Centrifuge Biofuge fresco	Heraeus, Hanau, Hanau
DNA-agarose gel electrophoresis chamber	Bio-Rad, Munich
Eagle eye (Mididoc)	Herolab, Wiesloch
ELISA plate reader	Anthos labtec, Wals, Austria
FACS Calibur	Becton-Dickinson, Heidelberg
Hyper processor (for processing films)	Amersham, Freiburg
Incubator for bacteria	Melag, Berlin
Incubator for cell culture	Labotec, Goettingen
Invert microscope DM-IL	Leica, Bensheim
LSM710 (laser scanning microscope)	Zeiss, Goettingen

Master cycler (PCR cycler)	Eppendorf, Hamburg,
Magnetic stirrer 3000	Heidolph, Keilheim
Microscope DMBRE	Leica, Bensheim
Microwave	Phillips, Wiesbaden
Photocassette	Amersham, Freiburg
Ph-Meter-761 Calimatic	Knick, Berlin
Photometer Ultraspec III	Amersham, Freiburg
Pipettus-Akku	Hirschmann, Eberstadt
Pipettes	Eppendorf, Hamburg
Powersupply PS 9009	GIBCO, Darmstadt
Rotor GSA	Kendro, USA
Rotor SW34	Kendro, USA
Rotor SW41 Ti	Beckman Coulter, Krefeld
Steril bank	Heraeus, Hanau
Sonicator Sonoplus	Bandelin, Berlin
Tabletop centrifuge	Heraeus, Hanau
Transferapparatus Mini Trans-Blot®	Bio-Rad, Munich

Thermo-mixer	Eppendorf, Hamburg
Ultrasound homogenizer	Bandelin Electronik
Water-bath	Julabo, Seelbach
Weighing scale RC210 D	Sartorius, Goettingen
Whirlmixer Vortex Genie	Si Inc., New York, USA

2.1.2 Miscellaneous Material

Cell culture flasks 25cm^2, 75cm^2	Greiner, Frickenhausen
Cell culture 96-well, 24-well, 6-well plates	Greiner, Frickenhausen
Centrifugal concentrators Vivaspin 6ml, 20ml	Vivascience, Hannover
Cryovials	Greiner, Frickenhausen
Coverglass	R. Langenbrinck, Emmendingen
Dako pen	DakoCytomat., Glostrup, Denmark
Electroporation cuvettes	Eugentec, Seraing, Belgium
Falcon tubes 15ml, 50ml	Greiner, Frickenhausen
Glass slides	R. Langenbrinck, Emmendingen
Hyperfilm ECL	Amersham, Freiburg

Needles	BD Biosciences, Heidelberg,
Nitrocellulose membrane Hybond ECL	Amersham, Freiburg
Parafilm	American Nat. Can., Greenwich, Great Britain
Petriplates	Greiner, Frickenhausen
Pipette tips	Sarstedt, Numbrecht
Sterile filter 0,2µm	Renner, Darmstadt
Syringes	BD Biosciences, Heidelberg
Trans-well migration (Boyden) chambers 48 well	Neuroprobe, Gaithusberg, USA
WhatmanTM 3MM paper	Scleicher & Schull, Dassel

2.1.3 Chemicals and Reagents

Acetic acid	Riedel-de Haen, Seelze
Acetone	Fluka, Buchs, Switzerland
Agarose	Sigma, Steinheim
Ammonium persulphate (APS)	GIBCO, Darmstadt
Ampicillin sulphate	Calbiochem, Darmstadt
Bactoagar	Fluka, Buchs, Switzerland

Bio-Rad, Munich Bradford reagent	Bio-Rad, Munich
Biotin-X-NHS	Calbiochem, Darmstadt
Bovine Serum Albumin (BSA)	PAA, Pasching, Austia
Brij 96	Fluka, Buchs, Switzerland
Bromo phenol blue	Merck, Darmstadt
Calcium chloride	Merck, Darmstadt
CFSE	Invitrogen, Darmstadt
Chloroform	Riedel-de Haen, Seelze
Coomassie R-250	Merck, Darmstadt
Crystal violet	Sigma, Steinheim
Dimethyl formamide	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Dynasore (dynamin 1 inhibitor)	Santacruz, Heidelberg
Embedding medium Neg-50	R.-A. Scientific, Kalamanzoo, USA
Ethanol	Riedel-de Haen, Seelze
Ethidium bromide	Merck, Darmstadt
Ethylenediamine tetraacitic acid (EDTA)	Sigma, Steinheim
Foetal Calf Serum (FCS)	PAA, Pasching, Austria

Formaldehyde (37%)	Merck, Darmstadt
G418 sulphate	PAA, Pasching, Austria
Gelatine (cold water fish skin)	Merck, Darmstadt
Glucose	Merck, Darmstadt
L-Glutamine	AppliChem, Darmstadt
Glycerine	Roth, Karlsruhe
Glycine	GERBU, Gaiberg
HEPES	GERBU, Gaiberg
HiPerfect-Reagent for transfection	Quiagen, Hilden
Hydrochloric acid (HCl)	Riedel-de Haen, Seelze
Hygromycin	PAA, Pasching, Austria
Immersion oil	Zeiss, Goettingen
Isopropanol	Fluka, Buchs, Switzerland
Laminin-5	K. Miyazaki, Yokohoma, Japan
Magnesium carbonate	Merck, Darmstadt
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Merck, Darmstadt

Milk powder	Roth, Karlsruhe
Matrigel (ECM gel)	Sigma, Steinheim
Methanol	Riedel-de Haen, Seelze
Mowiol (4-88)	Calbiochem, Darmstadt
N,N,N'N'-Tetramethylenediamine (TEMED)	Sigma, Steinheim
Paraformaldehyde	Sigma, Steinheim
Penicillin	Sigma, Steinheim
Phenylmethylsulphonylfluoride (PMSF)	Sigma, Steinheim
Phorbolmyristateacetate (PMA)	Sigma, Steinheim
Potassium acetate	Sigma, Steinheim
Potassium carbonate	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Potassium dihydrogenphosphate	Merck, Darmstadt
Potassium tetrathionate	Merck, Darmstadt
Protease Inhibitor Cocktail Tablets	Roche Diagnostics, Mannheim
Protein G Sepharose 4 Fast Flow	Amersham Biosciences, Freiburg
Rotipherose Gel 30 (Acrylamide-mix)	Roth, Karlsruhe

Rhodamine DHPE	Invitrogen, Darmstadt
RPMI 1640	GIBCO, Darmstadt cell culture
Silver nitrate	Roth, Karlsruhe
Sodium acetate	Merck, Darmstadt
Sodium azide	AppliChem, Darmstadt
Sodium carbonate	AppliChem, Darmstadt
Sodium chloride	Fluka, Buchs, Switzerland
Sodium hydrogen phosphate	Merck, Darmstadt
Sodium dodecyl sulphate (SDS)	GERBU, Gaiberg
Sodium hydrogen carbonate	AppliChem, Darmstadt
Sodium hydroxide	Riedel-de Haen, Seelze
Sodium pyruvate	Merck, Darmstadt
Sodium thiosulphate	Merck, Darmstadt
SP-Dio_{18}(3) dye for exosome labeling	Invitrogen, Darmstadt
Tris	Roth, Karlsruhe
Triton-X-100	Sigma, Steinheim
Trypan bue	Serva, Heidelberg

Trypsin	Sigma, Steinheim
Trypton	AppliChem, Darmstadt
Tween 20	Serva, Heidelberg
Yeast Extract	GIBCO, Darmstadt

2.1.4 Standard buffers and solutions

Bicarbonate buffer	15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6
Blot buffer	25 mM Tris, 192mM Glycine,0.1% SDS, 20% Methanol
Ethidium Bromide	0.01% (w/v) in water. Store in dark.
Freezing medium	10% DMSO in FCS
HEPES buffer	25mM HEPES, 150 mM NaCl, 5mM MgCl2, 1 mM PMSF, Protease inhibitors
6x Laemmli-buffer	350mM Tris, pH6.8, 10% (w/v) SDS, 36% (w/v) Glycerine, 0.01% (w/v) Bromophenol blue
LB medium	10g peptone, 5g yeast extract, 10g NaCl. Make volume to 1l. Add 15g agar for LB plates.

Running buffer for SDS-PAGE (10X)	1%SDS (w/v), 144g Glycine, 30g Tris. Make volume to 1l with bidest water.
PBS	137 mM NaCl, 8.1mM Na_2HPO_4, 2.7 mM KCl, 1.5mM KH_2PO_4, pH 7.4
PBG	PBS, 0.2% gelatin from fresh water goldfish, 1%BSA
Stripping / acid wash buffer	PBS-HCl pH2.5
Stripping buffer for western blots	62.5 mM Tris-HCl (pH 6.8), 2% SDS. ,0.1 M 2-Mecaptoethanol
TAE buffer	242g Tris base, 57.1ml Glacial acetic acid, 100ml 0.5M EDTA pH 8.0. Make volume to 1l and adjust pH to 8.5

2.1.5 Enzymes

Restriction enzymes	MBI Fermentas, St. Leon-Rot
Taq polymerase	MBI Fermentas, St. Leon-Rot
T4 Ligase	MBI Fermentas, St. Leon-Rot
Calf Intestinal alkaline phosphatase (CIAP)	MBI Fermentas, St. Leon-Rot

2.1.6 Kits

Quiaquick gel extraction kit	QUIAGEN, Hilden
Quiaquik Midiprep kit	QUIAGEN, Hilden

ECL Western Blotting Detection Reagents	Amersham, Freiburg

2.1.7 Size markers

GeneRulerTM 100bp DNA-Ladder Plus	MBI Fermentas, St. Leon-Rot
GeneRulerTM 1Kb DNA-Ladder Plus	MBI Fermentas, St. Leon-Rot
PagerulerTM Prestained Protein Ladder	MBI Fermentas, St. Leon-Rot

2.1.8 Antibodies

2.1.8.1 Primary Antibodies

Antibody	**Company**
α6β4 (clone B5.5)	Matzku et al., 1989
CD4 (clone Ox35)	European Association of Animal Cell Cultures
CD8 (clone Ox8)	European Association of Animal Cell Cultures
CD9; clone B2C11	BD Biosciences, Heidelberg; Developmental studies Hybridoma bank
CD11a	BD, Heidelberg, Germany
CD11b (clone Ox42)	European Association of Animal Cell Cultures
CD11c (clone Ox41)	European Association of Animal Cell Cultures
CD13	Chang et al, 2005
CD18	BD Biosciences, Heidelberg
CD29; (clone FW4.10.1)	BD Biosciences, Heidelberg, Developmental studies Hybridoma bank
CD31	BD, Heidelberg, Germany

CD44 (clone Ox50)	European Association of Animal Cell Cultures
CD44v6 (clone A2.6)	Matzku et al., 1989
CD49c ; clone Ralph3.1	BD Biosciences, Heidelberg, Developmental studies Hybridoma bank
CD49d	BD Biosciences, Heidelberg
CD49e	BD Biosciences, Heidelberg
CD49f	Abcam, Cambridge, U.K
CD54	BD Biosciences, Heidelberg
CD56	BD Biosciences, Heidelberg
CD61	BD Biosciences, Heidelberg
CD62L	BD Biosciences, Heidelberg
CD63	BD Biosciences, Heidelberg
CD81	BD Biosciences, Heidelberg
CD104	BD Biosciences, Heidelberg
CD106	BD Biosciences, Heidelberg
CD151	Claas et al., 2005
Caveolin	BD Biosciences, Heidelberg
Clathrin	Calbiochem, Darmstadt
D6.1	Matzku et al., 1998
EWI-F	Orlicky et al., 1998
ISN2	Santa Cruz, Heidelberg, Germany
Lamp1	BD Biosciences, Heidelberg
rab5	BD Biosciences, Heidelberg
rab7	Santa Cruz, Heidelberg, Germany
Transferrin receptor (Ox26)	European Collection of Animal Cell Culture

2.1.8.2 Secondary Antibodies and Reagents

Anti-mouseIgG-APC	BD Biosciences, Heidelberg
Anti-mouseIgG-PE	Dianova, Hamburg
Anti-mouseIgG-HRP	Rockland, Gilbertsville, PA, USA
Anti-goatIgG-Cy3	BD Biosciences, Heidelberg
Anti-goatIgG-Cy2	BD Biosciences, Heidelberg
Anti-rabbitIgG-Cy3	BD Biosciences, Heidelberg
Anti-rabbitIgG-Cy2	BD Biosciences, Heidelberg
Anti-rabbitIgG-HRP	Rockland, Gilbertsville, PA, USA
Anti-GuineapigIgG-PE	Dianova, Hamburg
Anti-GuineapigIgG-biotin	Rockland, Gilbertsville, PA, USA
Streptavidin-PE	Dianova, Hamburg
Streptavidin-HRP	Sigma, Steinheim
Streptavidin-Cy3	Dianova, Hamburg
Streptavidin-APC	Dianova, Hamburg

2.1.9 Expression vector

All the chimeric constructs were cloned into pcDNA3.1(+) plasmid with hygromycin resistance.

2.1.10 Bacterial strain

E.coli DH5α	Genotype: F^-, Φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-,mk^+), *pho*A, *sup*E44, *thi*-1, *gyr*A96, *rel* A1, λ^- (Invitrogen, Darmstadt, Karlsruhe)

2.1.11 Cell lines

BSp73AS (AS)	a non-metastasizing rat pancreatic adenocarcinoma line (Matzku et. al, 1983)
BSp73ASML	A highly metastatic rat pancreatic adenocarcinoma line Matzku et al., 1983
AS-Tspan8	AS cells transfected with pcDNA3.1(+) neo containing Tspan8 cDNA (Claas et. al, 1998)
AS-Tspan8/CD151c	AS cells transfected with pcDNA3.1(+) Hygro containing Tspan8/CD151c cDNA
AS-Tspan8/CD151n	AS cells transfected with pcDNA3.1(+) Hygro containing Tspan8/CD151n cDNA
AS-Tspan8/CD151c+n	AS cells transfected with pcDNA3.1(+) Hygro containing Tspan8/CD151c+n cDNA
AS-Tspan8/CD9c	AS cells transfected with pcDNA3.1(+) Hygro containing Tspan8/CD9c cDNA
AS-Tspan8/CD9n	AS cells transfected with pcDNA3.1(+) Hygro containing Tspan8/CD9n cDNA
AS-Tspan8/CD9c+n	AS cells transfected with pcDNA3.1(+) Hygro containing Tspan8/CD9c+n cDNA
AS-Tspan8-CD104	Herlevsen et al., 2003

Rat aorta endothelial cell line (RAEC)	Isolated from Wistar rats, Cell lining, Berlin
Fibroblasts (Fb)	Isolated from lungs of BDX rats with $NiSO_4$
Stromal cells	Isolated from lymph nodes of BDX rats

2.1.12 Rat strain

BDX rats were bred in animal facility of University of Heidelberg under pathogen-free conditions and were provided sterile food and water ad libitum. Rats in the age 9-11wk were used for experiments.

2.2 Methods

2.2.1 Molecular Biology

2.2.1.1 Bacteria

For all bacterial work DH5α was used. DH5α was cultured in liquid Luria Bertani (LB) medium or on solid LB-agar plates containing 60µg/ml ampicillin for selection. Transformations were carried out by either Eppendorf, Hamburg electroporator according to manufacturer's instructions, or by heat shock method as widely used. Briefly, 50µl of competent DH5α from -80˚C were kept on ice. 50ng of plasmid DNA was added and incubated for 10 min on ice. This bacteria-plasmid mix was placed at 42˚C for 45 seconds, and then placed back on ice for 2 min to reduce damage to bacteria. 1ml of LB was added and the bacteria incubated at 37˚C with agitation for 45min to 1hr. After this, around 100µl of the resulting culture were plated on an LB-agar selection plate and incubated 12-16h to get colonies.

2.2.1.2 Plasmid-DNA- Preparation

Plasmid miniprep and midiprep were done with Quiagen mini and midi-prep kits according to supplier's instructions.

2.2.1.3 Generation of chimeric constructs

The following chimeric Tspan8 cDNA were generated: Tspan8/CD151c and Tspan8/CD9c with exchange of AA 198-235 by AA 214-253 of CD151 or AA 189-226 of CD9; Tspan8/CD151n and Tspan8/CD9n with exchange of AA 1-80 by AA 1-85 of CD151 or AA 1-82 of CD9; CD151 and CD9, where the large extracellular loop (AA 86-205 of CD151 and AA 82-200 of CD9) were exchanged by AA 81-197 of Tspan8 (Tspan8/CD151c+n, Tspan8/CD9c+n). All construct were cloned into pcDNA3.1(+) using the restriction sites Kpnl and XhoI or Apa1 in a two step cloning procedure using the wt Tspan8, CD9 or CD151 cDNA as templates and overlapping primers (TableA). After ligating the cDNA of chimeric molecules with the pcDNA3.1 (+) hygro, these plasmids were used for transforming E coli DH5α. which were grown on ampicillin containing LB-agar plates. Plasmids from bacterial colonies was digested as well as checked with PCR to verify correct insert-size. Promising plasmids were sent for sequencing to confirm the absence of any mutations. Large quantities of the plasmid with chimeric cDNA insert were produced by plasmid midi-prep (Quiagen).

AS cells were stably transfected with these chimeric cDNA. Transfected AS clones were selected by growth in RPMI1640 supplemented with glutamine, antibiotics, 10% FCS and 750µg/ml G418 under limiting dilution. Transfection with INS2-siRNA followed standard procedures as suggested by the supplier (Quiagen, Hildesheim, Germany). Efficiency of RNA silencing was monitored after 48h by WB. AS, transfected AS cells, aortic ring derived endothelial cell line (RAEC), Lung fibroblast and lymph node stroma lines derived from rats were maintained in RPMI1640/ 10% FCS. Confluent cultures were detached by EDTA and split.

Table 2.1 Scheme of PCRs for generating chimeric constructs

Construct	1 PCR :Primers (Primer number as in table B)	Template	2 PCR	Template
Tspan8WT	Tspan8_KpnKoz_fw (12)			
	Tspan8_Xho1_rev (9)	Tspan8cDNA		
CD151 WT	CD151_Kpn_fw (4)			
	CD151_Apa1_rev (5)	CD151 cDNA		

CD9 WT	CD9_Kpn1-Koz_fw (7)			
	CD9_Xho1_rev (11)	CD9 cDNA		
Tspan8/CD151c	Tspan8_CD151ec2_fw (3)		Fragment PCR1	
	CD151_Apa1_rev (5)	CD151 cDNA	Tspan8_Kpn_fw (1)	Tspan8 cDNA
Tspan8/CD151n	CD151_ Tspan8_fw (6)		Fragment PCR1	
	Tspan8_Xho1_rev (9)	Tspan8cDNA	CD151_Kpn_fw (4)	CD151 cDNA
Tspan8/CD151c+n	CD151/ Tspan8_fw (6)		Fragment PCR1	
	CD151_Apa1_rev (5)	Tspan8/CD151c	CD151_Kpn_fw (4)	CD151 cDNA
Tspan8/CD9c	Tspan8/ctCD9_fw (10)		Fragment PCR1	
	CD9 XhoI_rev (11)	CD9 cDNA	Tspan8_KpnI-Koz_fw (12)	Tspan8cDNA
Tspan8/CD9n	CD9 KpnI-Koz fw (7)		Fragment PCR1	
	CD9/D6.1Aec2 rev (8)	CD9 cDNA	D6.1A_XhoI_rev (9)	Tspan8cDNA
D6.1A/CD9n+c	D6.1A/ctCD9_fw (10)		Fragment PCR1	
	CD9 XhoI_rev (11)	CD9 cDNA	CD9_Xho1_ rev (11)	D6.1A/CD9n

Table2.2 List of primers used for PCRs for generating chimeric constructs

Primer	**Sequence in 5'-3' orientation**
(1)D6.1A Kpn1_fw	5'-AAAGGTACCGCCACCATGGATTACAAGGATG
	ACGACGATAAGGCAGGTGTCAGTGGCTGTTTA-3'
(2)D6.1A_Xho1_rev	5'-GTCTCGAGTCATTTGCTTCCAATTTGGCA-3'
(3)D6.1A_CD151ec2_fw	5'-CCTGTCTTTCTCTGATAAAATCCTTCATTCAAGCACCTG-3'
(4)CD151_Kpn1_fw	5'-AAAGGTACCGCCACCATGGATTACAAGGATGACGACGATAAG GGGGAATTCAACGAGAAGAAG-3'
(5)CD151_Apa1_rev	5'-AAAGGGCCCTCAGTAGTGCTCCAGCTTGAG-3'
(6)CD151/D6.1A_fw	5'-GCTGTGCCACTTTCAAGGAGAGTCGCTGCATGCTTCTCTT-3'
(7)CD9_ KpnI-Koz_fw	5'-CAGGTACCGCCACC ATG GGCCGGTCAAAGGAG-3'
(8)CD9/D6.1Aec2_rv	5' GAGAAGCATGCAGCGACTCTC TTGTACAGCTCCACAGCA-3'

(9)D6.1A XhoI_rv	5'-GTCTCGAGTCATTTGCTTCCAATTTGG-3'
(10)D6.1A/ctCD9_fw	5'-ACAGAAAGAGACTATTTTTCGTTCAAGGTGTAGTAACCT- 3'
(11)CD9 XhoI_rv	5'-GTCTCGAGCTAGACCATTTCTCGGCTCCT-3'
(12)D6.1A KpnI-Koz_fw	5'-CAGGTACCGCCACCATGGCAGGTGTCAGTGGC-3'

2.2.1.4 Dephosphorylation of plasmid DNA

Recircularization of vector DNA was minimized by removing the 5´-phosphate residues from both termini of the linear, double-stranded plasmid DNA with calf intestinal alkaline phosphatase (CIAP, MBI Fermentas). The reaction was incubated at 37˚C for 30 min and then stopped by heating at 85°C for 15 minutes.

2.2.1.5 Plasmid ligation

For ligation of cDNA fragments into the plasmid T4 ligase with its lgation buffer (MBI Fermentas) was used. 200 ng of digested and dephosphorylated pcDNA3.1 (+) plasmid DNA were used. The amount of insert was calculated according to a molar ration 3:1 (insert: vector). The reactions were incubated for 1 hour at room temperature. To inactivate the Ligase the reaction was transferred to 70˚C for 15 min. To determine the efficiency of ligation, 5 µl of the reaction mix were run on an agarose gel and visualised with ethidium bromide. If the ligation was successful, 1-5 µl of the reaction mix was used for transformation of E.coli.

2.2.1.6 siRNA transfection

For the transfection of BSp73AS-Tspan8 cells, $5x10^4$ cells per well were plated in a 24-well plate. Next day, the cells were transfected with the INS2 siRNA (Quiagen, Hilden) according to manufacturer's protocol (HiPerfect-Reagent-Protocol, Quiagen, Hilden). The efficiency of transfection was checked by western blots 24, 48 and 72h after transfection.

2.2.2 Cell biology

2.2.2.1 Cell culture

Eukaryotic cells were kept in RPMI 1640- medium, containing 10% heat inactivated fetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin and maintained at 37˚C, 95% humidity and 5% CO_2. For passaging, cells were detached with EDTA.

2.2.2.2 Cryopreservation of eukaryotic cells

$1x10^7$ cells were harvested, washed once with fresh medium and resuspended in ice-cold FCS/10% DMSO. Cells were kept overnight at -80˚C and transferred to liquid nitrogen thereafter.

2.2.2.3 Transfection of eukaryotic cells

$6x10^5$ AS cells were seeded the day before in a 6 well-plate. Next day, cells were transfected at ~80% confluency with Lipofectamine 2000 according to supplier's instructions.

2.2.2.4 Recloning of transfected cells by limited dilution

Transfected cells were selected for drug resistance and checked by FACs for expression of the transgene. Limited dilutions of 1 or 3 cells per well were carried out in a 96 well-plate. Cells were grown in the presence of $1x10^6$ freshly prepared rat thymocytes as feeder cells. Clones were checked by FACs and used for a second round of dilution to ensure a single cell clone.

2.2.2.5 Adhesion assay

Cells were CFSE-labeled (5µM) for 20min in serum free medium. After this, cells were washed twice in RPMI containing FCS and incubated for another 30min in fresh medium at 37˚C. Following this, $5x10^4$ cells were seeded on BSA-, fibronectin (FN, 1µg/ml) - or laminin5 (Ln5, 2µg/ml) - coated plates or were seeded on a monolayer of RAEC. Where indicated, cells were PMA (10^{-8}M) treated and/or the medium contained antibody (10µg/ml).

After 2h at 37˚C, plates were washed and adhesion was determined in Fluoroskan Ascent multiplate reader (excitation: 490 nm, emission: 518 nm).

2.2.2.6 Migration assays

Subconfluent monolayers in a 6 well plate were scratched with a pipette tip. Wound healing was evaluated after 24h-72h by light microscopy. Transwell migration was evaluated using Boyden chambers with 8µm pore size membranes. Cells (1x106/ml, RPMI/0.1% FCS) were added to the upper chamber. Cells were stimulated by PMA (10-8M) or were pre-incubated with antibody (10µg/ml). RPMI1640/20% FCS was added to the lower chamber. After 12h incubation, migrated cells, on the lower surface of the filter were fixed with ice cold methanol and stained with crystal violet. Cells were dissolved in 10% acetic acid measuring absorbance at 595nm.

2.2.2.7 Matrigel assay

Matrigel was thawed overnight at 4˚C. The following day, it was diluted 1:1 with cold RPMI-1640 (serum free). 100µl of this dilution was plated carefully in wells of a 24- well plate. It was kept at 37˚C for 30min to solidify. Later, $5x10^4$ cells were seeded in 200µl RPMI-1640 over the matrigel in 24-well plates. Cable formation was evaluated after 48h by light microscopy.

2.2.2.8 Immunofluorescence

$3x10^5$ cells were grown on cover slips for 48hrs. Prior to staining, cells were fixed with 4% paraformaldehyde for 15min at RT, permeabilized with 0.2% tween in PBS-1%BSA, washed, blocked with 0.2% gelatin (freshwater goldfish) in PBS-1%BSA 15min and incubated with primary antibodies (2-10µg/ml, 60min, 4˚C). Cover slides were rinsed and incubated with a fluorochrome-conjugated secondary antibody (60min, 4˚C). After blocking, and incubation with a second, dye-labeled antibody (60min, 4˚C), cover slides were washed and mounted in elvanol.

For internalization studies, cells were first incubated with a dye-labeled primary antibody or the primary antibody for 30min (4˚C), washed and incubated for the indicated time points

with PMA 100nM at 37˚C for internalization. Following this, cells were immediately placed back on ice, fixed, permeabilized and thereafter incubated with secondary, dye-labeled antibody (60min, 4˚C).

Where indicated, pre-starved cells (10h in serum free medium) were incubated with the dynamin inhibitor Dynasore (80µM in RPMI1640, 5h, 37˚C). After transfer on ice and blocking (PBS/0.5%BSA/0.2%gelatine), cells were incubated with the primary antibody, washed and incubated for 30min at 37˚C in the presence of PMA and Dynasore. Cells were washed, stripped, fixed, permeabilized and incubated with the dye-labeled secondary antibody and DAPI. Digitized images were generated using a Leica DMRBE microscope or a Carl Zeiss LSM710 confocal microscope and software Carl Zeiss Axioview Rel. 4.6.

2.2.2.9 Flow cytometry

1-3×10^5 cells per sample were taken in a 96-well U bottom plate. Trypsinized cells were allowed to recover (2h, 37˚C, RPMI1640/10% FCS). After washing with FACS buffer (PBS, 1% FCS, pH7.4), cells were incubated with the primary antibody (1-5µg/ml) for 30min at 4˚C, washed twice with FACS buffer and thereafter incubated with secondary, fluorochrome-conjugated antibody (0.3-0.5µg/ml) for 30min, 4˚C. For double fluorescence, cells were incubated with a blocking antibody prior to incubations with the next set of primary and secondary antibody incubations. After 3 washes in FACS buffer, samples were acquired and analyzed with the FACS Calibur (BD, Heidelberg, Germany). Where mentioned, cells were fixed in 1% formalin (20min, 4˚C), washed and permeabilized with 0.2% tween In FACS buffer before incubations with the antibodies.

For internalization studies, cells were stained with primary antibody on ice, washed and incubated at 37˚C for 30min. Afterwards, the cells were brought back to ice and the non-internalized, surface bound antibody was stripped off, with PBS-HCl, pH2.5 (2washes, 5min, 4˚C) Cells were then fixed and permeabilized followed by incubation with the secondary, dye labeled antibody (30min, 4˚C). Stripped samples were compared to non-stripped samples to quantify internalization.

2.2.2.10 Exosome Isolation

Supernatants from cells grown in serum free conditions were collected after 24-72h of culture, and processed through a density gradient centrifugation at 4˚C: 500g for 10min, 2000g for 10min, 5000g for 20 min, 10,000g for 30min, followed by 2.5h of ultracentrifugation at 100,000g using SW41Ti rotor in Beckman Coulter ultracentrifuge. The exosome pellets were re-suspended in PBS, protein amount measured using Bradford assay and stored at -80˚C until use.

2.2.2.11 Fluorescent-labeling of exosomes

Exosomes were labeled directly or indirectly from cells. For indirect labeling, confluent cells in flasks were washed with PBS to remove all the FCS. Thereafter cells were labeled by incubation in medium (devoid of serum) containing either DHPE (Lissamine™ rhodamine B 1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt. (rhodamine DHPE); excitation at 560 nm, emission at 586 nm) or SP-DioC_{18} (3) (excitation at 497nm, emission at 513nm) dyes diluted (1:10,000) for 30min in the incubator. After this, the cells were washed twice to get rid of excess of dye. Cells were then kept in culture for 48h without serum for later collecting exosomes.

For direct labeling, 1mg of exosomes was labeled in 200µl of PBS containing DHPE or SP-DioC_{18} (3) at 1:10,000 dilution in PBS for 15min RT. After this, the labeled exosomes were washed twice with exosome-depleted FCS so that the free dye binds to proteins in FCS. Exosomes were centrifuged down in between washes and after the last wash in PBS. The labeled exosome-pellet was then resuspended in PBS and stored at -80˚C till further use.

2.2.2.12 FACS for evaluating exosomes subpopulations

1µl Latex beads (4µm diameter) with activated aldehyde groups were pre-coated with D6.1 or CD9 antibody (10µg/ml) overnight at 4˚C with agitation. Following this, the remaining free aldehyde groups were quenched by incubation with PBS-100mM Glycine for 30min, RT. Then, 20µg of DHPE labeled exosomes were incubated with the pre-coated beads for 2hr at RT. The bead-unbound exosomes were collected and bound to uncoated beads. After washing with PBS-1% FCS (exosome depleted), the exosome-bead complex was incubated

with primary antibody (5µg/ml) for 30min, RT, washed, and incubated with the APC-conjugated secondary antibody (0.3µg/ml) for 30min, RT. After washing, samples were acquired and analyzed with FACS Calibur.

2.2.3 Animal experiments

2.2.3.1 In vivo exosome targeting

SP-DioC_{18} (3) (Invitrogen, Darmstadt) labeled 250µg exosomes were injected intravenously (i.v.) in the tails of BDX rats (9-11wk). After 24h, the rats were sacrificed, organs extracted, and meshed through cotton gauze to prepare cell suspensions for FACS analysis. Parts of organs were also frozen in liquid nitrogen in Neg-50 embedding medium for later analysis with confocal microscopy.

2.2.3.2 Ex vivo exosome binding

Organs from healthy BDX rats were extracted and the cell suspensions from various organs were co-incubated with 20µg/ml of DHPE-labeled exosomes for 6h. Cells were then distributed in U-form 96well plates for FACS. Cells were washed, fixed and permeabilized prior to incubation with primary antibody (30min, 4˚C), washed and incubated with the APC-conjugated secondary antibody (30min, 4˚C).

2.2.3.3 Cell and tissue preparation

Heparinized peripheral blood was collected by heart puncture. Peripheral blood mononuclear cells (PBL) were collected after Ficoll-Hypaque gradient centrifugation. Peritoneal exudate cells (PEC) were collected by flushing the peritoneal cavity with 10ml PBS/heparin. Bone marrow cells (BMC) were collected from femur and tibia by flushing the bones with 5ml PBS. Spleen and LN cells (SC, LNC) were obtained by pressing the organs through fine gauze. Liver, lung, pancreatic and submandibular gland cells were collected from minced tissue after 3x30min treatment with a collagenase, dispase mix. Alternatively, solid organs were shock frozen in liquid nitrogen.

2.2.3.4 Statistical analysis

All assays, which were statistically evaluated, were repeated at least 3 times. P-values <0.05 (two tailed Student's t-test and Anova) were considered significant.

2.2.4 Protein Biochemistry

2.2.4.1 Biotinylation

Cells were washed in cold HEPES buffer and, incubated with 0.1mg/ml water-soluble Biotin-X-NHS in HEPES buffer on a shaking platform (30min, 4°C). Cells were washed with PBS-200mM Glycine and lysed (HEPES buffer, 1% Lubrol or 1% Brij96, 1mM PMSF and protease inhibitor cocktail) for 60min, 4°C. Exosomes were biotinylated similarly, after quenching and washing, ultracentrifugation steps (100,000g for 2,5h) were required to pellet down the exosomes.

2.2.4.2 Immunoprecipitation (IP)

Lysates from cells were centrifuged for 15min at 15000g to remove unsolubilized material and cell nuclei. 1mg for cell lysates or 100µg exosomal lysate was immunoprecipitated with corresponding antibody (2µg/mg of lysates or 200µl hybridoma) O/N at 4°C, followed by incubation with 5% proteinG-Sepharose (1h) with rotation. Thereafter, the complexes were washed four times with lysis buffer. After the last wash, all the liquid was removed through a 35g-needle attached to a vacuum pump to ensure minimal background. Complexes were resuspended in Laemmli buffer and boiled for 5min at 95˚C. Sepharose beads were separated by a quick spin at 15000g for 15sec and the supernatant was subjected to SDS-PAGE.

2.2.4.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

For electrophoretic separation of protein samples the 'Mini-Protean II' system was used for discontinuous SDS-PAGE. 5ml of separating gel (375mM Tris pH8.8, 0.1%SDS, 10-12% acrylamide-bisacrylamide, 0.1%TEMED (v/v), 0.1% (w/v) ammonium persulphate) was overlaid with 2ml of stacking gel (375mM Tris pH6.8, 0.1%SDS, 4% acrylamide-bisacrylamide, 0.1%TEMED (v/v), 0.1% (w/v) ammonium persulphate). After complete

polymerization, gels were loaded and run in gel running buffer at a constant voltage of 120V. Gels were either silver stained or subjected to western blot analysis.

2.2.4.4 Western blotting

After SDS-PAGE, protein gels were equilibrated for 10min in transfer buffer. Nitrocellulose membranes (Amersham, Freiburg) and 3MM Whatman paper were equilibrated as well. For protein transfer, the gel was placed on Whatman paper, followed by nitrocellulose and another layer of Whatman paper. The wet transfer was carried out in transfer buffer at a constant voltage of 30V O/N at 4˚C.

After transfer had been completed, the membranes were blocked for 1h at RT with 5% (w/v) fat-free milk in PBST (PBS, 0.1% (v/v) Tween-20). Antibody incubations were carried out for 1h at RT with hybridoma supernatant or purified antibody in PBST-milk. Membranes were then washed three times, 5min each in PBST and then probed with horse radish peroxidase (HRP)-conjugated secondary antibody (diluted 1:10000 in PBST) for 1h at RT, followed by additional three washing steps. Biotinylated proteins were detected with Streptavidin-peroxidase. Detection was done by chemiluminiscence using the ‘ECL Western blotting detection reagents’ and ‘ECL radiography films’ from Amersham, Freiburg.

2.2.4.5 Silver staining of protein gels

After separation of proteins by SDS-PAGE, gels were fixed overnight in 30% ethanol/ 10% acetic acid and sensitized for 45min in 0.3% potassium tetrathionate, 0.5M potassium acetate, 30% ethanol. This was followed by 6 washes, totally for 1h with bidest water. Gels were stained with 0.2% silver nitrate for 1-2h, rinsed with bidest water and developed for upto 40min in developer (3% potassium carbonate, 31µl $Na_2S_2O_3$-$5H_2O$ (10%), 75µl formalin (37%) per 250ml). The reaction was stopped by adding 330mM Tris/ 2% acetic acid and gels were kept in bidest water.

2.2.4.6 Sucrose Gradient Ultracentrifugation

800µl of cell lysates were mixed with 800µl of 80% sucrose (prepared in 1x HEPES) yielding 1.6ml lysates in 40% sucrose which was placed at the bottom of a 4ml

ultracentrifuge tube. Over this was laid, 1.6ml of 30% sucrose in HEPES followed by a layer of 5%sucrose in HEPES on the top. The tubes were carefully placed in a SW41Ti rotor and centrifuged in an ultracentrifuge (Beckman Coulter, Krefeld) at 30000 rpm/100,000g at 4 ˚C for 13h. Afterwards, 12 equal fractions were collected from each tube and either used for SDS PAGE directly, or used for IPs after pooling several fractions and their dialysis.

3. Results

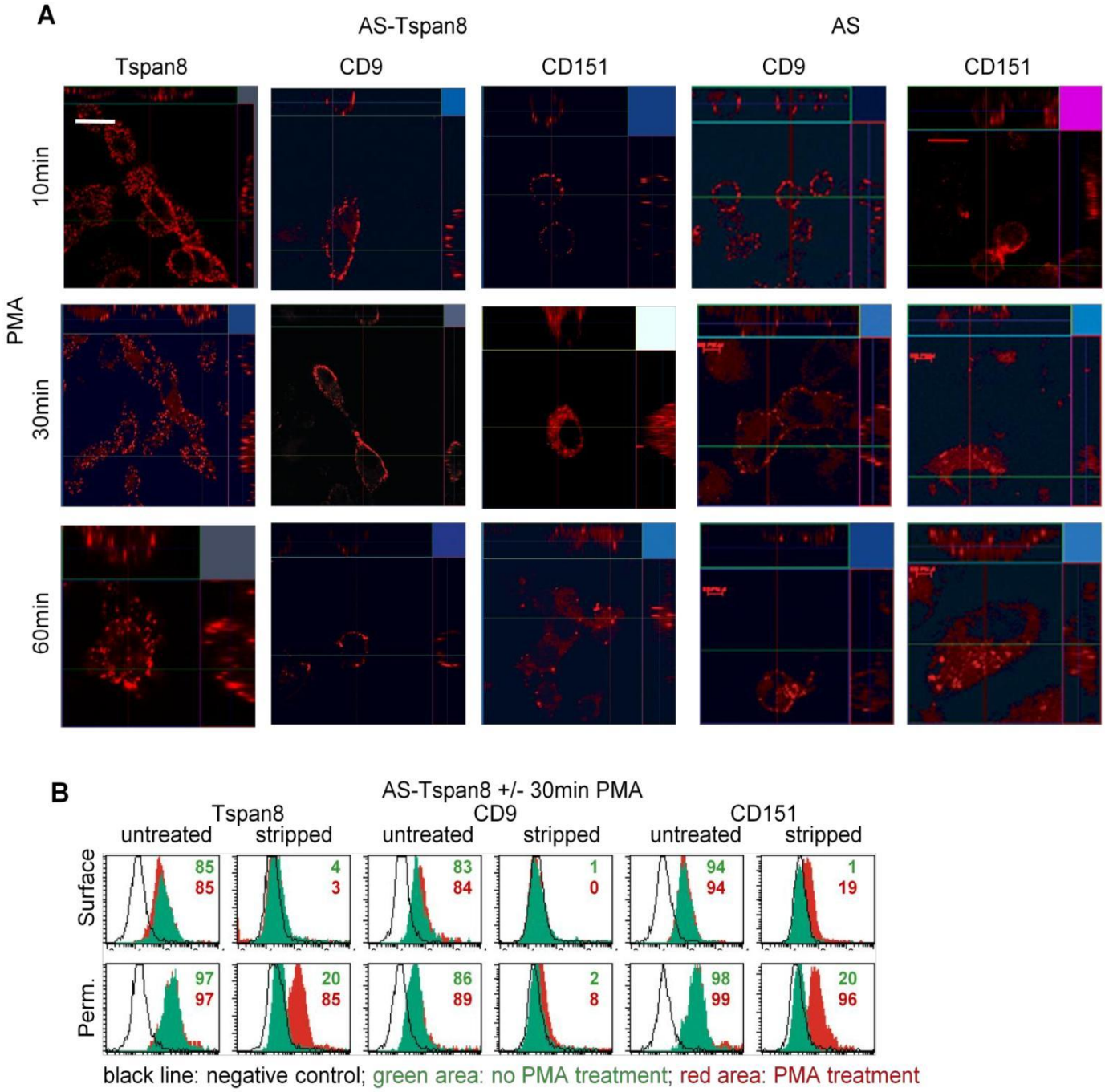
It was recently described in our lab, that a tumor cell line overexpressing the tetraspanin Tspan8 delivers exosomes that induce resting endothelial cell (EC) activation and angiogenesis (Gesierich et al., 2006**)**. Notably, AS-Tspan8 cells express besides Tspan8, the tetraspanins CD9, CD81, CD151 at comparable levels, which are also expressed on exosomes derived from AS, AS-Tspan8-CD104. But, only AS-Tspan8 exosomes promoted angiogenesis in vivo and not AS or the AS-Tspan8-CD104 derived exosomes. This selective activity of exosomal Tspan8 and Tspan8-associated CD49d prompted us to explore the feature of Tspan8 internalization.

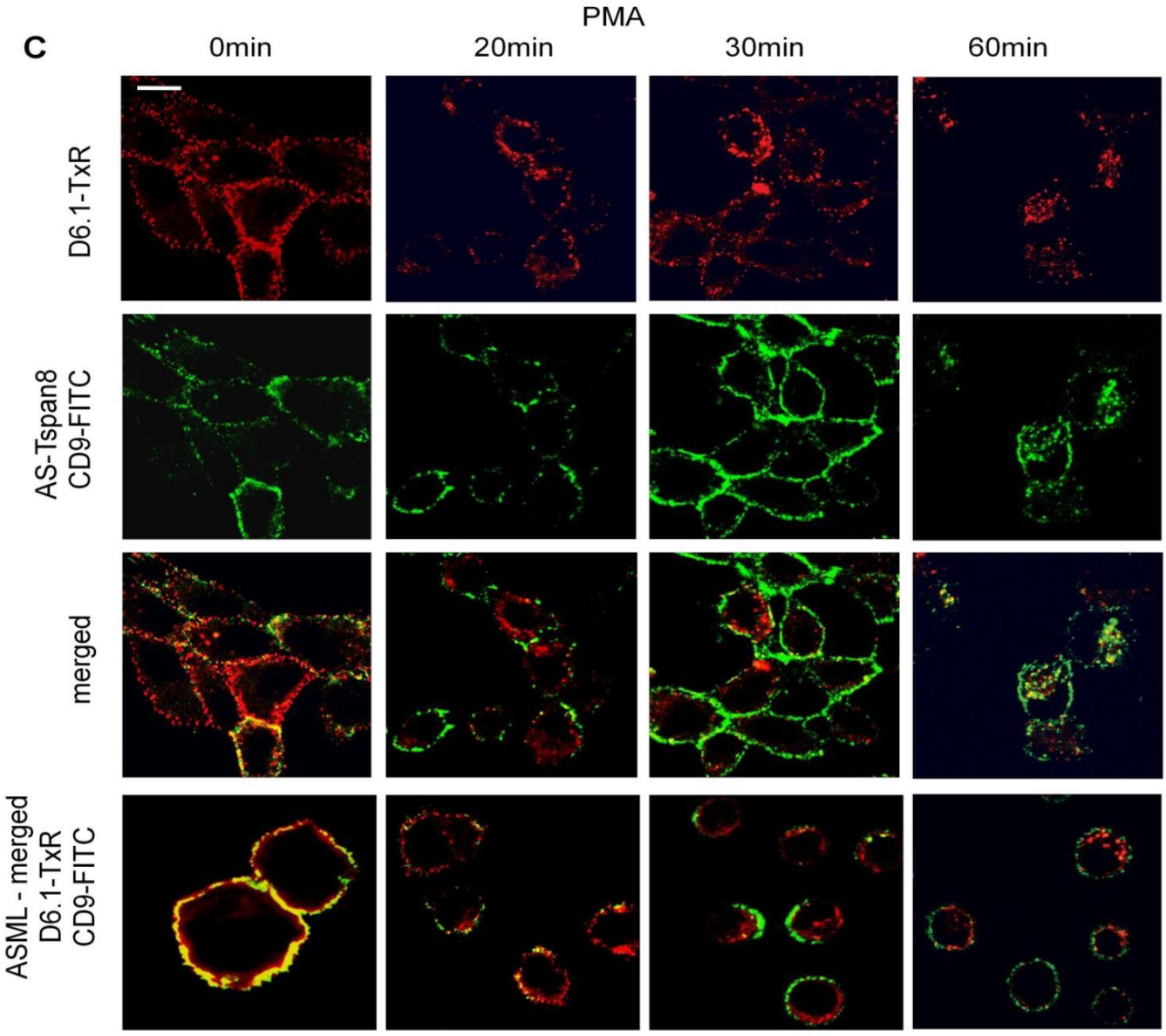
3.1 Tspan8, CD9 and CD151 get internalized differently

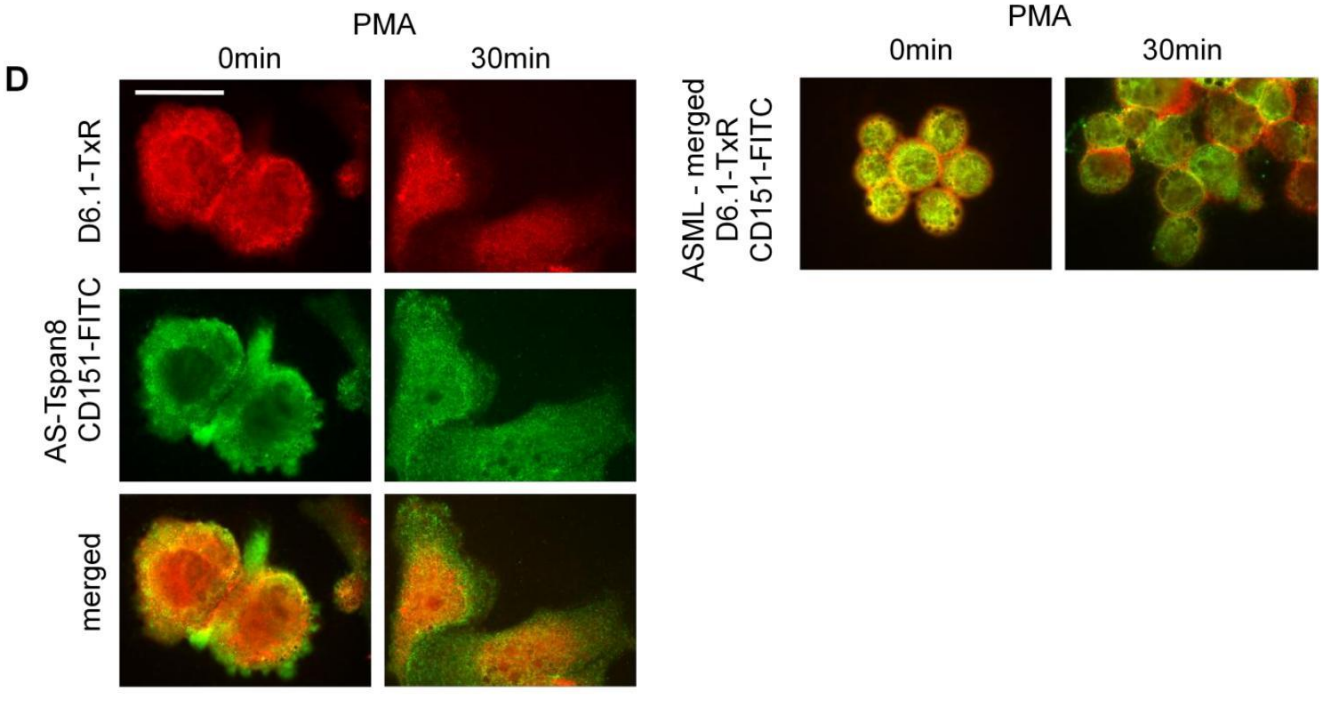
Exosome delivery is known to be strengthened under stress conditions (Parolini et. al, 2009). Keeping this in mind, and also to avoid selective endocytosis of individual tetraspanins which can be triggered by antibody cross-linking, we evaluated internalization in PMA-treated cells. AS and AS-Tspan8 cells were incubated with D6.1 (anti-Tspan8), anti-CD9 or anti-CD151 on ice. After washing, cells were treated for 10-60min with PMA (10^{-8}M) in RPMI at 37^{o}C, 5%CO_2. Thereafter, cells were fixed, permeabilized and stained with the secondary antibody. CD151 becomes internalized within 10-30min of PMA-treatment whereas, Tspan8 internalization requires 30min with complete internalization seen after 60min. CD9 remains at the plasma membrane until 30min of PMA-treatment and is partially internalized after 60min (Fig. 1A).

To confirm the process of tetraspanin internalization, recovery of surface-bound antibody was measured by flow cytometry after internalization for 30min and stripping in non-permeabilized versus permeabilized PMA-treated AS-Tspan8 cells. Surface staining of Tspan8, CD9 and CD151 was largely lost after stripping. However, Tspan8 and CD151, but not CD9, were recovered in PMA-treated, stripped and permeabilized AS-Tspan8 cells. This confirmed internalization of CD151, partial internalization of Tspan8 and persisting membrane expression of CD9 (Fig.1B). These findings also pointed towards Tspan8 internalization proceeding independent of the tetraspanin web. To further support this hypothesis, AS-Tspan8 and ASML cells were PMA-treated and thereafter stained with D6.1-TxR and anti-CD9-

FITC. Indeed, Tspan8 co-localizes with CD9 in untreated, but not in PMA-treated AS-Tspan8 cells (Fig.1C). The same finding accounted for co-localization of Tspan8 with CD151, however, co-localization in untreated cells was weaker than co-localization of Tspan8 with CD9 (Fig.1D). The rapid internalization of Tspan8 was unexpected, because Tspan8 does not contain a properly located internalization motif. We therefore generated Tspan8 chimeric molecules to explore, which region of Tspan8 accounts for the more rapid internalization compared to CD9.




C
PMA
0min
20min
30min
60min
D6.1-TxR
AS-Tspan8 CD9-FITC
merged
ASML - merged D6.1-TxR CD9-FITC


D
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0min
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ASML - merged D6.1-TxR CD151-FITC

Figure 1. PMA-induced internalization of CD9, CD151 and Tspan8: (A) AS and AS-Tspan8 cells were incubated at 4°C with anti-CD9, anti-CD151, D6.1 (anti-Tspan8), washed, treated for 10, 30 or 60min with PMA (37°C) and stained with the secondary dye-labeled antibody. The tetraspanin distribution was evaluated by confocal microscopy, including sagittal sections (scale bar: 10µm). (B) AS-Tspan8 cells, stained with D6.1, anti-CD9 and anti-CD151, were treated for 30min with PMA. Surface and permeabilized staining are shown. Where indicated, cells were permeabilized or first the membrane bound staining was stripped and then cells permeabilized to reveal internalization. Overlays with the negative control and the mean percentage of stained cells are presented. (C and D) Co-localization of Tspan8 with CD9 (C) and CD151 (D) during PMA-treatment of AS-Tspan8 and ASML cells was evaluated by fluorescence microscopy after staining with D6.1-TxR and CD9-FITC or CD151-FITC. Single fluorescence staining and overlays are shown (scale bar: 10µm).

3.2 PMA induced internalization of chimera vs. Tspan8

Six chimeric constructs were created as follows: Tspan8/ CD151n, Tspan8/CD9n where the n terminus of Tspan8 was exchanged with amino acids of CD9 or CD151 n-terminus. Tspan8/ CD151c, or Tspan8/CD9c where the c-terminus of Tspan8 was exchanged for c-terminus of CD9 or that of CD151 and; Tspan8/CD151n+c, Tspan8/CD9n+c where both the c- and n-termini were exchanged with either those of CD9 or CD151. The cDNA of these constructs were cloned into pcDNA3.1 (+) vector which also contained hygromycin resistance cassette. Sequences for Tspan8, CD9, CD151 and the chimeric molecules are mentioned in Table1.

Tspan8	MAGVSGCLKYSMFFFNFLFWVCGTLILGLAIWLRVSKDGKEIITSGDNGTNPFIAVNILIAVGSIIMVLGF LGCCGAVKESRCMLLLFFIGLLLILLLQVAAGILGATFKSESSRILNETLYENAKLLSETSNEAKEVQKAMI AFQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIKELVEKNIIIVIGIAFGLAV IEILGLVFSMVLYCQIGSK
CD151	MGEFNEKKATCGTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYISLLASSTYLATAYILVVAGV VVMVTGVLGCCATFKERRNLLRLYFILLLIIFLLEIIAGILAYVYYQQLNTELKENLKDTMIKRYHQSGHE GVTNAVDKLQQEFHCCGSNNSRDWRDSEWIRSGEADSRVVPDSCCKTVVTGCGKREHASNIYKVEGGCITK LESFIQEHLRVIGAVGIGIACVQVFGMIFTCCLYRSLKLEHY
CD9	MPVKGGSKCIKYLLFGFNFIFWLAGIAVLAIGLWLRFDSQTKSIFEQETNHSSFYTGVYILIGAGALMMLV GFLGCCGAVQESQCMLGLFFGFLLVIFAIEIAAAVWGYTHKDEVIKELQEFYKDTYQKLRNKDEPQRETL KAIHMALNCCGIAGGVEQFISDICPKKQVLESFQVKSCPDAIDEVFHSKFHIIGAVGIGIAVVMIFGMIFSMIL CCAIRRSREMV
Tspan8/CD151n	*MGEFNEKKATCGTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYISLLASSTYLATAYILV VAGVVVMVTGVLGCCATFKE*SRCMLLLFFIGLLLILLLQVAAGILGATFKSESSRILNETLYENAKLLSE TSNEAKEVQKAMIAFQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIKELVE KNIIIVIGIAFGLAVIEILGLVFSMVLYCQIGSK
Tspan8/CD151c	MAGVSGCLKYSMFFFNFLFWVCGTLILGLAIWLRVSKDGKEIITSGDNGTNPFIAVNILIAVGSIIMVLGF LGCCGAVKESRCMLLLFFIGLLLILLLQVAAGILGATFKSESSRILNETLYENAKLLSETSNEAKEVQKAMI AFQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIK*SFIQEHLRVIGAVGIGI ACVQVFGMIFTCCLYRSLKLEHY*
Tspan8/CD151n+c	*MGEFNEKKATCGTVCLKYLLFTYNCCFWLAGLAVMAVGIWTLALKSDYISLLASSTYLATAYILV VAGVVVMVTGVLGCCATFKE*SRCMLLLFFIGLLLILLLQVAAGILGATFKSESSRILNETLYENAKLLSE TSNEAKEVQKAMIAFQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIK*SFIQ EHLRVIGAVGIGIACVQVFGMIFTCCLYRSLKLEHY*
Tspan8/CD9n	*MPVKGGSKCIKYLLFGFNFIFWLAGIAVLAIGLWLRFDSQTKSIFEQETNHSSFYTGVYILIGAG ALMMLVGFLGCCGAVQE*SRCMLLLFFIGLLLILLLQVAAGILGATFKSESSRILNETLYENAKLLSETSN EAKEVQKAMIAFQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIKELVEKNII IVIGIAFGLAVIEILGLVFSMVLYCQIGSK

Tspan8/CD9c	MAGVSGCLKY**SMFFFNFLFWVCGTLILGLAIW**LRVSKDGKEIITSGDNGTNP**FIAVNILIAVGSIIMVLGFL** **GCC**GAVKESRCM**LLLFFIGLLLILLLQVAAGILGA**TFKSESSRILNETLYENAKLLSETSNEAKEVQKAMIA FQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIK***SKFHIIGAVGIGIAVVMI*** ***FGMIFSMILCCAIRRSREMV***
Tspan8/CD9n+c	***MPVKGGSKCIKYLLFGFNFIFWLAGIAVLAIGLWLRFDSQTKSIFEQETNHSSFYTGVYILIGAG*** ***ALMMLVGFLGCCGAVQE***SRCM**LLLFFIGLLLILLLQVAAGILGAT**FKSESSRILNETLYENAKLLSETSN EAKEVQKAMIAFQSEFKCCGLRFGAADWGKNFPDAKESCQCTGSDCESYNGENVYRTTCLSLIK***SKFHIIG*** ***AVGIGIAVVMIFGMIFSMILCCAIRRSREMV***

Table 1. Protein sequences of Tspan8, CD151, CD9 and the chimeric constructs. Blue: transmembrane regions; red: extracellular regions; black: intracellular regions; ***Large italic font:*** exchanged regions

AS cells were transfected with pcDNA3.1 (+) containing cDNA of Tspan8 or the Tspan8 chimeric constructs using Lipofectamine 2000. Limited dilution to get single stable clones was carried out under selection pressure. Expression levels were checked by FACS. A schematic presentation of the constructs is shown in Fig2A and expression of the chimeric Tspan8 molecules is shown in Fig2B. Tspan8 and the chimeric constructs were expressed at comparable levels in the AS cells, and thus could be used in further experiments for comparisons. We first evaluated whether these chimera may become internalized with different kinetics.

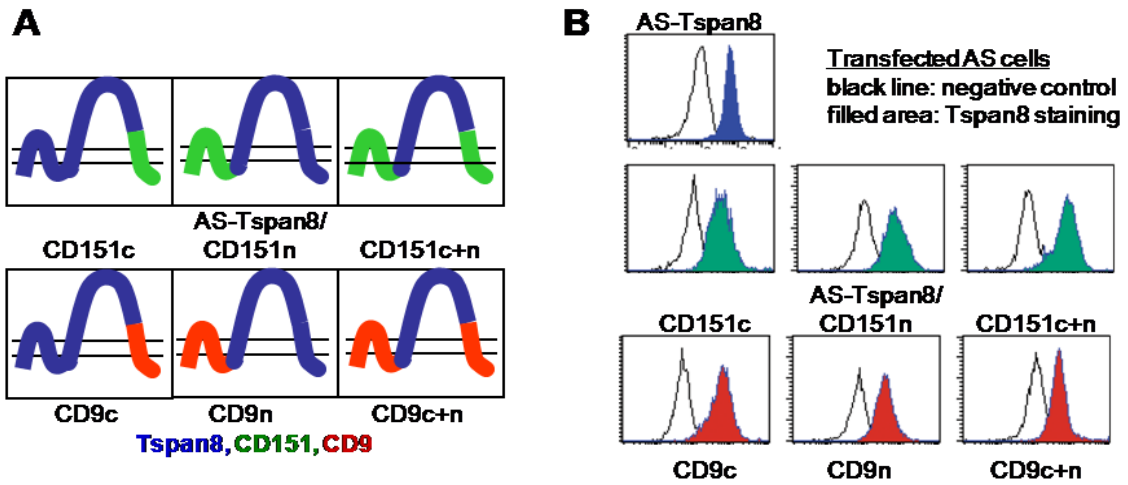
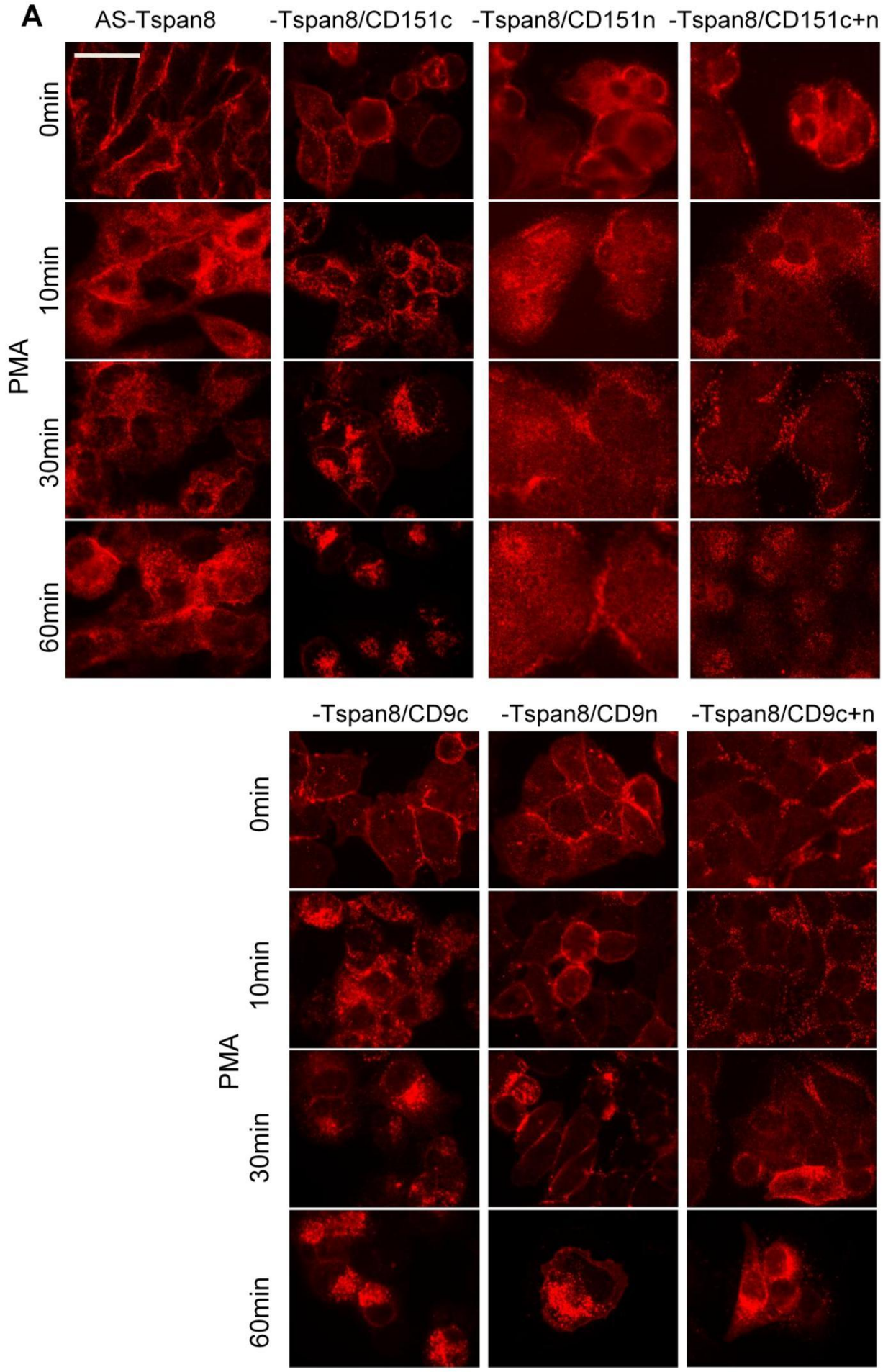


Figure.2.**Chimeric constructs and their expression** (A) Schematic representation of chimeric constructs. (B) Surface expression of the chimeric constructs in AS cells. Cells were stained with D6.1 (30min, 4˚C), washed with PBS and incubated with PE conjugated secondary Ab, washed and measured with FACS Calibur.

As revealed by D6.1-TxR staining, PMA-treatment and permeabilization, exchange of the N-terminal region particularly by that of CD9 was accompanied by retarded Tspan8 internalization (Fig.3A). The latter has been confirmed by flow cytometry, where after stripping hardly any intracellular Tspan8/CD9n was recovered, whereas internalization of Tspan8/CD151c was unimpaired (Fig.3B).

Evaluating co-localization of Tspan8 with CD9 after PMA-treatment confirmed exchange by the CD9 N-terminal and, less pronounced, the CD151-N-terminal region to promote persisting membrane expression of Tspan8 and co-localization of Tspan8 with CD9 in the plasma membrane. Instead, exchange of the Tspan8 C-terminal region by that of CD9 or CD151 had no effect on Tspan8 internalization and Tspan8 did not co-localize with CD9 after PMA-treatment (Fig.3C). The weaker co-localization of Tspan8 with CD151 was affected to a lesser extent by exchange of the Tspan8 C- or N-terminal region (data not shown). However, it should be mentioned that the CD151 C-terminal region, which contains a YxxΦ internalization motif, did not suffice to stabilize co-internalization of Tspan8 and CD151, which excludes a major contribution of the YxxΦ internalization motif of CD151 in Tspan8 internalization.


A
AS-Tspan8
-Tspan8/CD151c
-Tspan8/CD151n
-Tspan8/CD151c+n
PMA
0min
10min
30min
60min
-Tspan8/CD9c
-Tspan8/CD9n
-Tspan8/CD9c+n
PMA
0min
10min
30min
60min

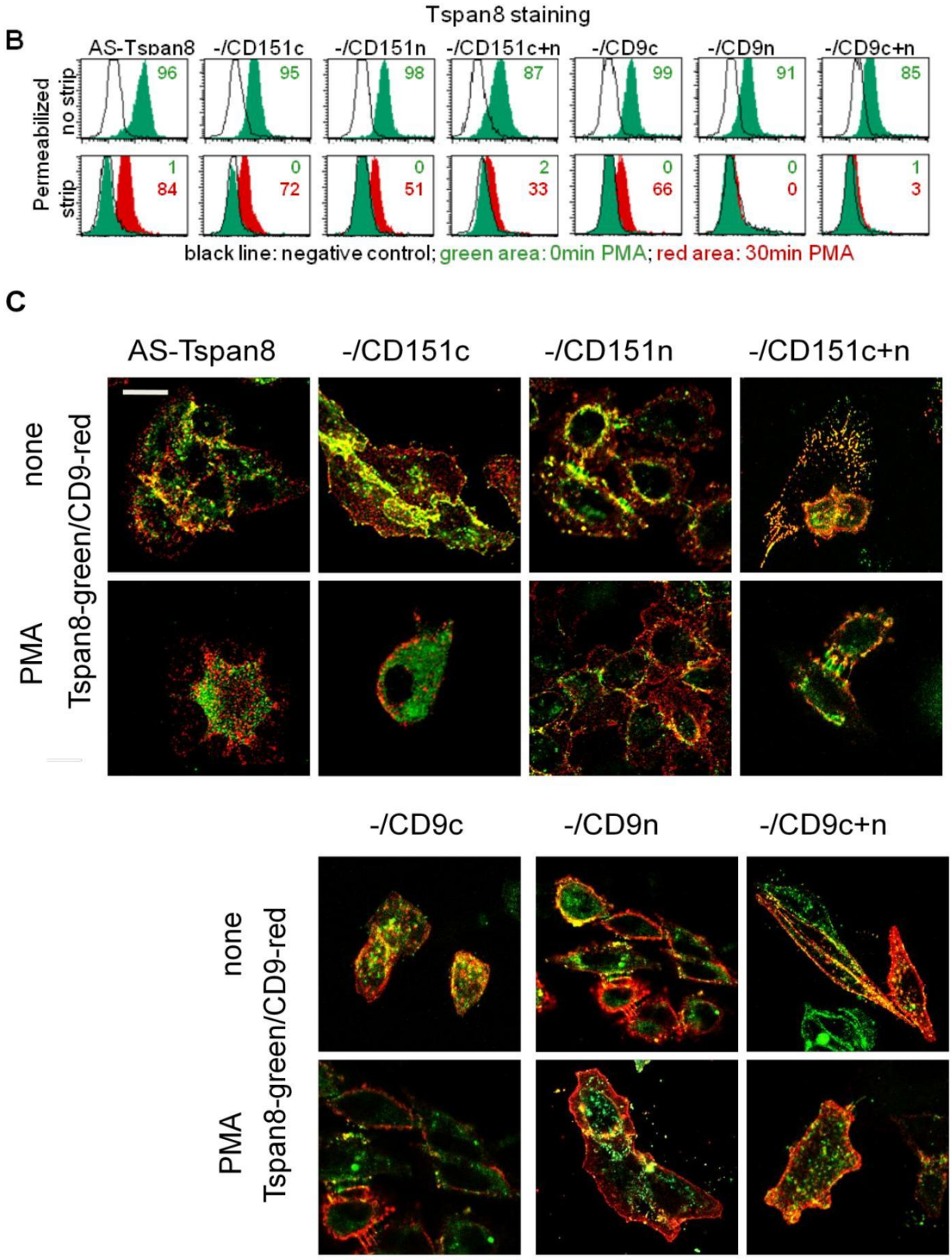


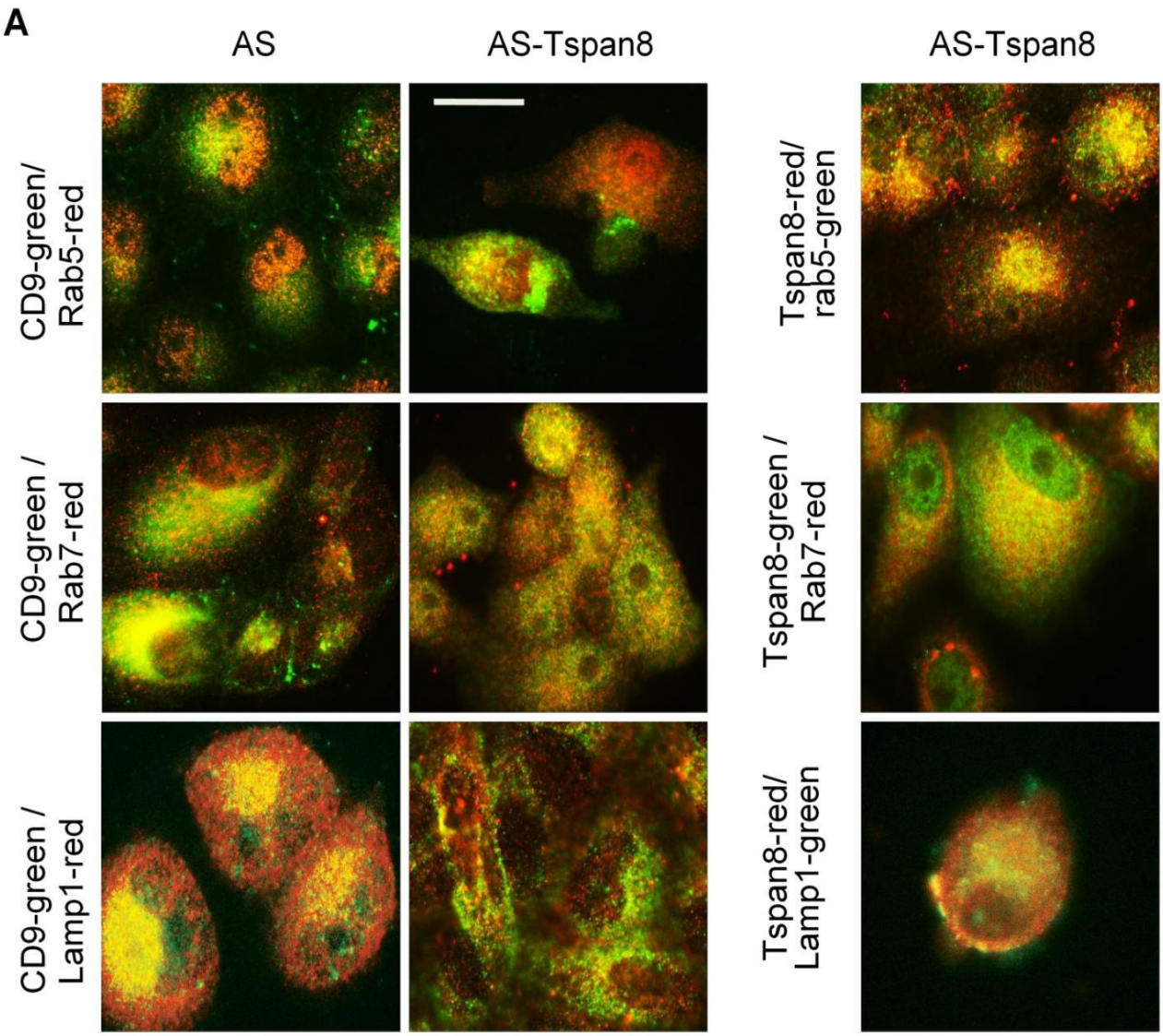
Figure 3. Internalization of chimeric molecules, and colocalization with CD9. (A) Time course of PMA-induced Tspan8-chimeric molecule internalization. Tspan8 localization was evaluated by fluorescence microscopy at 0-60 min of PMA-treatment (scale bar: 10μm). (B) Cells incubated with D6.1 (4°C) and treated, where indicated, with PMA for 0min or 30min (37°C); Cells were stripped where indicated, fixed, permeabilized and counterstained with anti-mIgG-PE. Overlays with the negative control and the mean percentage of stained cells are presented. (C) Co-localization of Tspan8 with CD9 in untreated and PMA- treated AS-Tspan8-chimeras was evaluated by confocal microscopy. Overlays of Tspan8-Cy2 and CD9-TxR staining are shown (scale bar: 10μm).

3.3 The route of internalization

3.3.1 Vesicular trafficking

Internalized membrane microdomains are recruited into early endosomes from where they can proceed to late endosomes. Alternatively, via ubiquitinylation and fusion with lysosomes proteins may become degraded. Early endosomes also can incorporate into MVB, migrate towards and fuse with the cell membrane such that the invaginated early endosomes are released as exosomes (de Gassart et al., 2004, Denzer et al., 2000, Stahl et al., 2002, van Niel et al., 2006). After PMA-treatment, Tspan8, distinct from CD9, mainly co-localized with the early endosomal marker Rab5, and rarely with the late endosomal marker Rab7. Tspan8 and CD9 also co-localized with LAMP1, which is found in late endosomes and synthetic pathway vesicles (Chapuy et al., 2008, Lakkaraju et al., 2008) (Fig.4A). Co-localization of Tspan8 with rab7 and Lamp1 was not significantly altered in AS cells expressing Tspan8 chimeric molecules. However, Tspan8/CD9n poorly and Tspan8/CD9c+n hardly co-localized with Rab5 (Fig.4B).

Taken together, PMA-treatment promotes Tspan8 internalization more efficiently than that of CD9 and co-localization of Tspan8 with CD9 and CD151 is not maintained. Also distinct from CD9, Tspan8 is mostly recovered in early endosomes. The analysis of Tspan8 chimeric molecules so far indicated that the Tspan8 N-terminal region might contribute to Tspan8 internalization. This led to addressing the question whether Tspan8 becomes internalized via molecules that selectively associate with Tspan8.


A
AS
AS-Tspan8
AS-Tspan8
CD9-green/
Rab5-red
Tspan8-red/
rab5-green
CD9-green /
Rab7-red
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Lamp1-red
Tspan8-red/
Lamp1-green

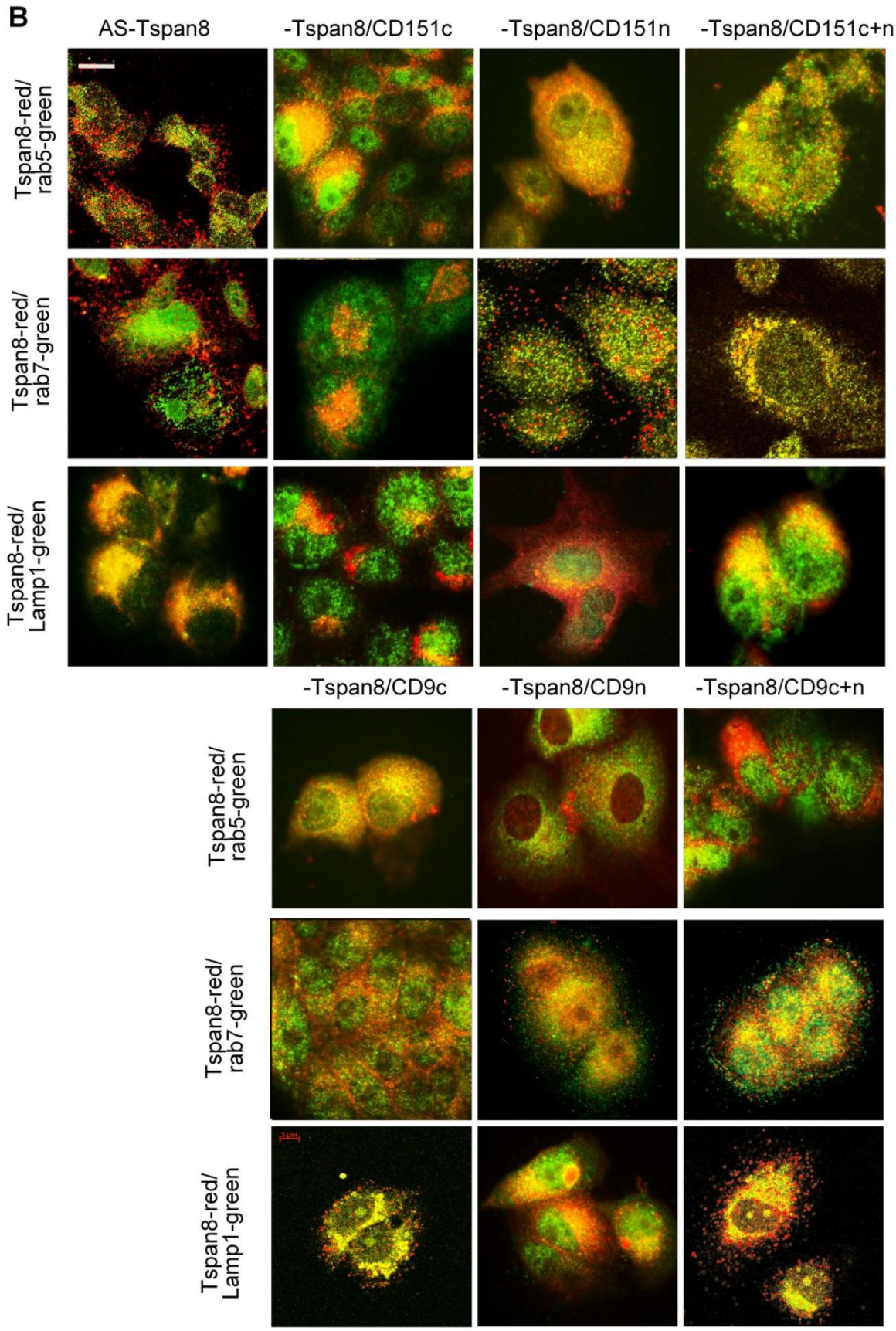
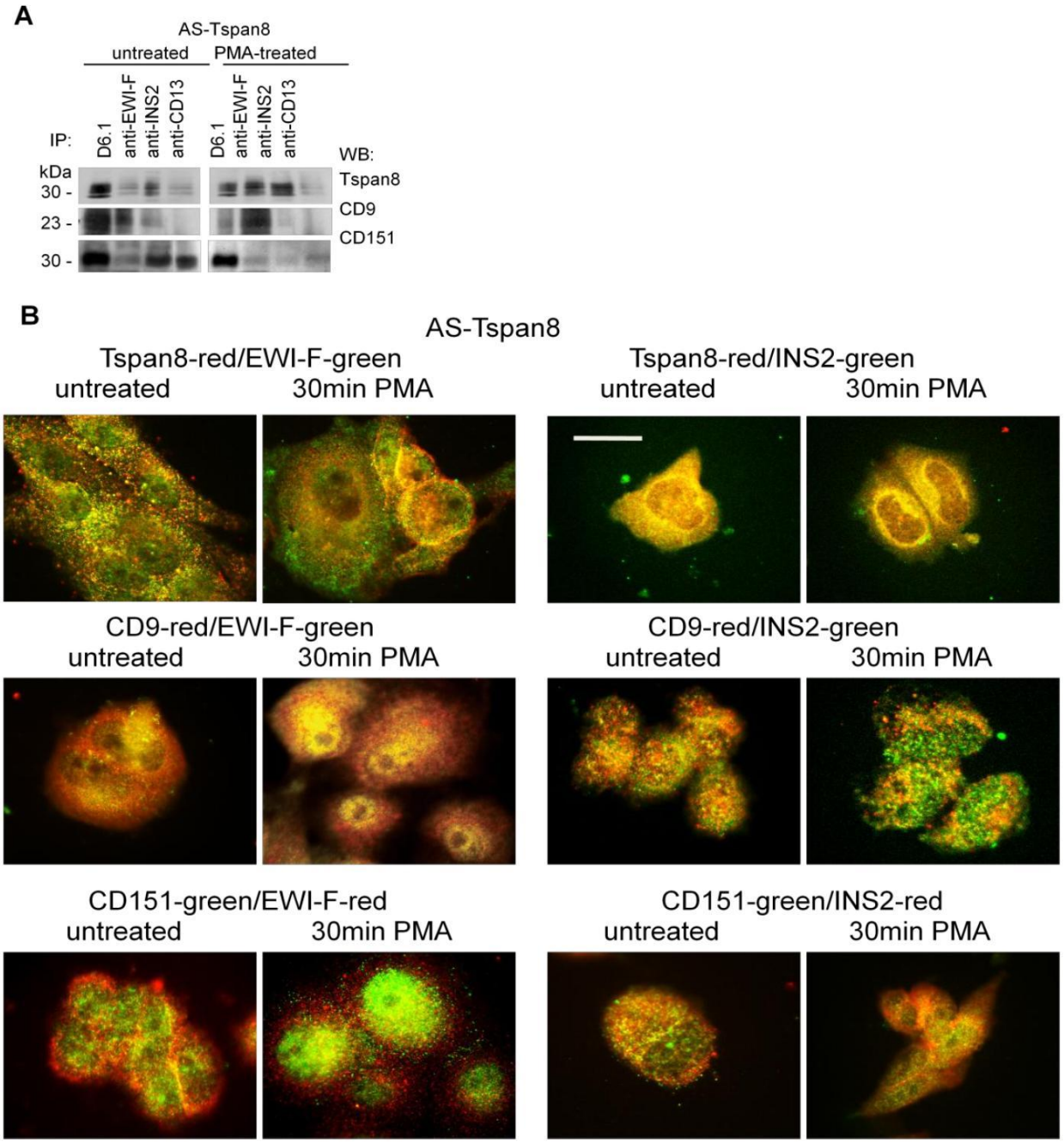


Figure 4: **Vesicular traffic of Tspan8 in PMA-treated cells:** (A) AS and AS-Tspan8 were PMA-treated (37˚C), fixed and permeabilized and stained with anti-CD9 or anti-Tspan8, the secondary dye-labeled antibody and anti-Rab5, anti-Rab7 and anti-Lamp1. (B) AS-Tspan8 and AS-Tspan8-chimeric cells were incubated with D6.1 (4˚C), PMA-treated (37˚C), fixed and permeabilized and stained with the secondary dye-labeled antibody and anti-Rab5, anti-Rab7 and anti-Lamp1. Merged views of fluorescence (A) or confocal (B) microscopy are shown (scale bar: 10μm).

3.3.2 Involvement of Intersectin-2

Tspan8 directly associates with EWI-F, the dipeptidase CD13 and INS2 (Claas et al., 2005, Le Naour et al., 2006, Claas et al, in prep.). These three molecules co-immunoprecipitate with Tspan8 after lysis in Brij96 and co-immunoprecipitation is maintained after PMA-treatment. A similar immunoprecipitation profile is seen with CD151, although the co-immunoprecipitation with INS2 becomes weaker after PMA-treatment. Instead, CD9 mostly co-immunoprecipitates with EWI-F. It also should be noted that Tspan8, though weakly co-localizing, co-immunoprecipitates with CD151 in untreated and PMA-treated AS-Tspan8cells, whereas co-immunoprecipitation of Tspan8 with CD9 becomes weak in PMA



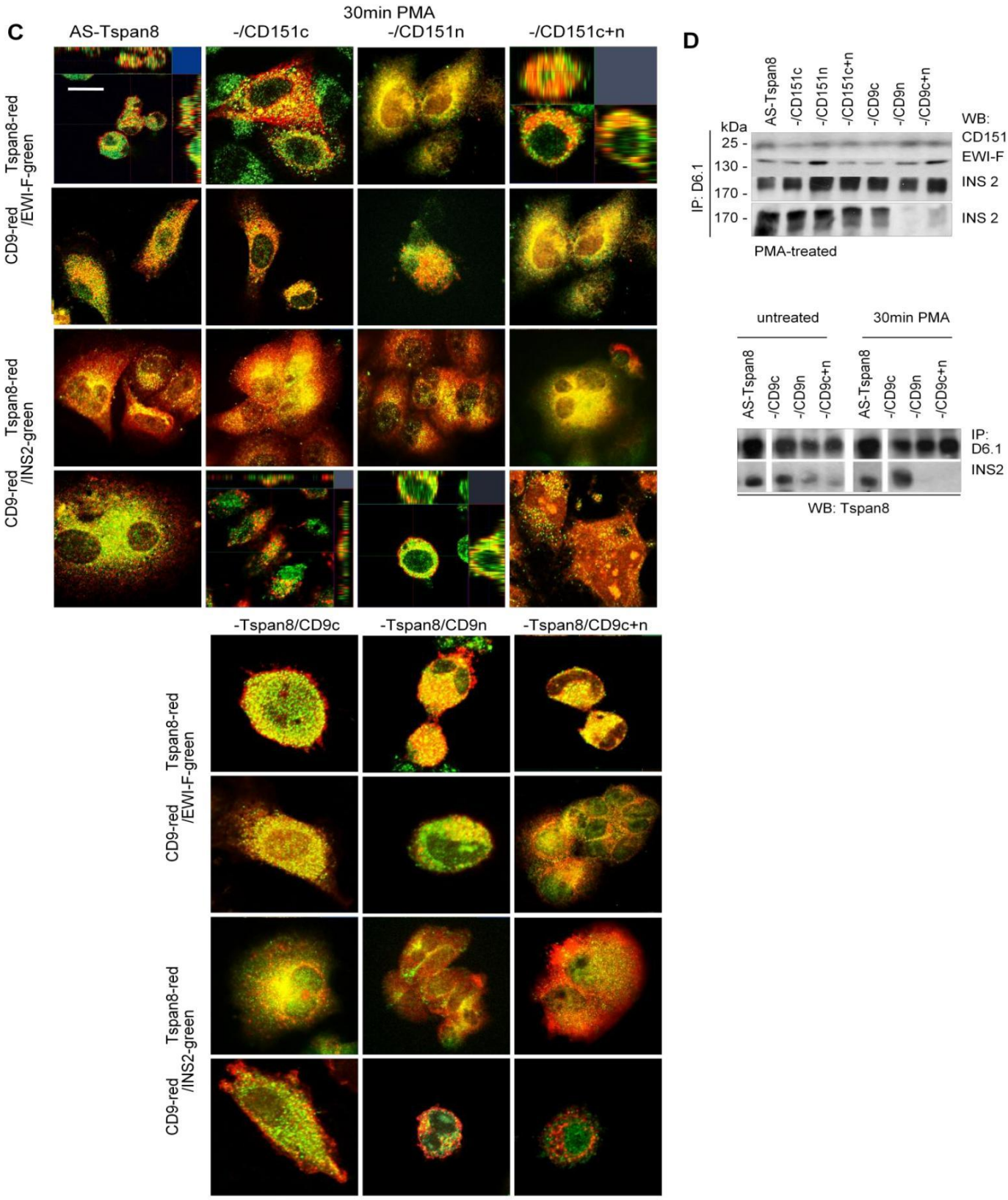


Figure 5. **Tspan8 associates and co-internalizes with INS2**: (A) Lysates of AS-Tspan8 cells were immunoprecipitated with D6.1, anti-EWI-F, anti-INS2 and anti-CD13. After SDS-PAGE separation and protein transfer, membranes were blotted with D6.1, anti-CD9 and anti-CD151. (B) AS-Tspan8 cells were PMA-treated and co-localization of Tspan8, CD9 and CD151 with EWI-F and INS2 was evaluated by fluorescence microscopy (scale bar: 10µm). Overlays of single fluorescence staining are shown. (C) Co-internalization of Tspan8 and CD9 with EWI-F and INS2 in PMA-treated AS-Tspan8-chimeras was evaluated by confocal microscopy (scale bar: 10µm).. Overlays of Tspan8 and CD9 staining with EWI-F and INS2 are shown. (D) Lysates of untreated and PMA-treated AS-Tspan8-chimeras were immunoprecipitated

with D6.1 or anti-INS2. Precipitates were separated by SDS-PAGE. After transfer, blots were developed with anti-CD151, anti-EWI-F and anti-INS2 or D6.1.

treated AS-Tspan8 cells (Fig.5A). Fluorescence microscopy confirmed co-localization of Tspan8, CD9 and CD151 with EWI-F. Co-localization of Tspan8, CD9 and, weakly, CD151 with EWI-F is maintained after PMA-treatment. The stronger co-localization of Tspan8 and the weaker one of CD151 with INS2 are maintained in PMA-treated AS-Tspan8 cells. Instead, weak co-localization of CD9 with INS2 is further reduced (Fig.5B). Co-localization of Tspan8 and CD9 with EWI-F during internalization is maintained in Tspan8-chimeras. This also accounts for the weak co-localization of CD9 with INS2. On the contrary, strong co-localization of Tspan8 with INS2 becomes weak in PMA-treated AS-Tspan8/CD9n chimeras (Fig.5C). All Tspan8 chimeric molecules weakly co-immunoprecipitate with CD151 and, though to a variable degree, with EWI-F. They also co-immunoprecipitate with INS2. However, after PMA-treatment, co-immunoprecipitation of INS2 with Tspan8 chimeras becomes weak and is no longer seen in AS-Tspan/CD9n and AS-Tspan8/CD9c+n chimeras. This was confirmed in the reverse setting, where INS2-immunoprecipitate of untreated cells contains little Tspan8/CD9n and Tspan8/CD9c+n. After PMA-treatment INS2 does not co-immunoprecipitate Tspan8/CD9n and Tspan8/CD9c+n, although comparable amounts of Tspan8 are precipitated by D6.1, which confirms reduced association of Tspan8/CD9n and Tspan8/CD9c+n with INS2 after PMA-treatment (Fig.5D).

INS2 being involved in clathrin-mediated endocytosis (Simpson et al., 1999), we next asked whether Tspan8, CD151 and CD9 are co-localizing or co-immunoprecipitating with clathrin and, for comparison, with caveolin. In untreated AS and AS-Tspan8 cells, the three tetraspanins strongly co-localize with clathrin, but co-localization of CD9 with clathrin becomes weak after PMA-treatment. Instead, co-localization of CD9 with caveolin is stronger after PMA-treatment (Fig.6A). Tspan8 does not or rarely co-localize with caveolin in PMA-treated AS-Tspan8 and AS-Tspan8-chimeras, but co-localizes with clathrin, except in AS-Tspan8/CD9n and AS-Tspan8/CD9c+n cells, where weak co-localization is mostly restricted to the cell membrane (Fig.6B). Co-immunoprecipitation confirmed that Tspan8 preferentially co-immunoprecipitates with clathrin. Instead, CD9 and CD151 co-immunoprecipitate with both clathrin and caveolin (Fig.6C). Anti-clathrin also immunoprecipitated chimeric Tspan8. However, co-immunoprecipitation became weak in PMA-treated AS-Tspan8/C9, particularly, AS-Tspan8/CD9c+n chimeras (Fig.6D). These data argued for the

N-terminal region of Tspan8 being essential for internalization as well as for a contribution of INS2 and clathrin.

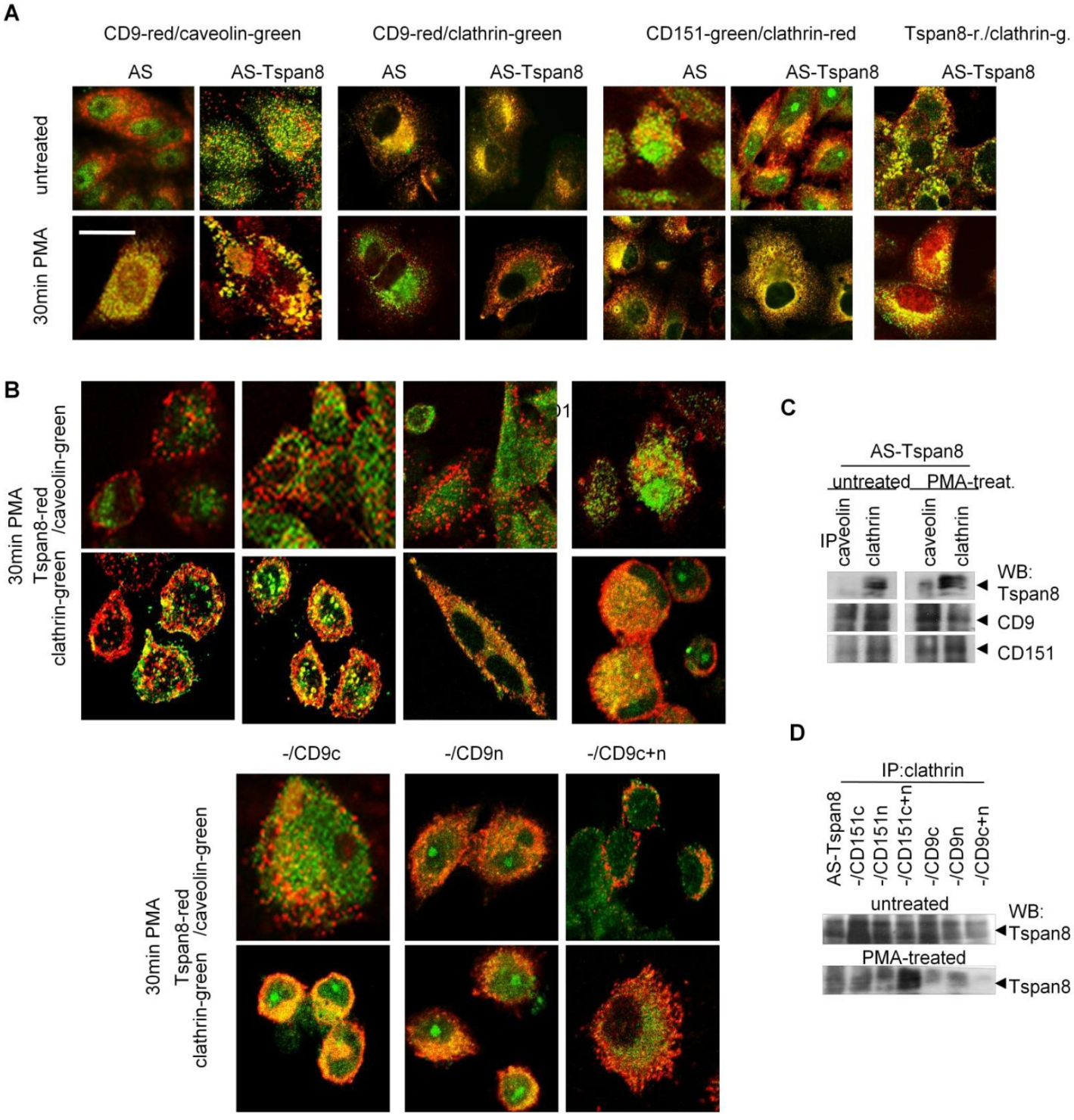
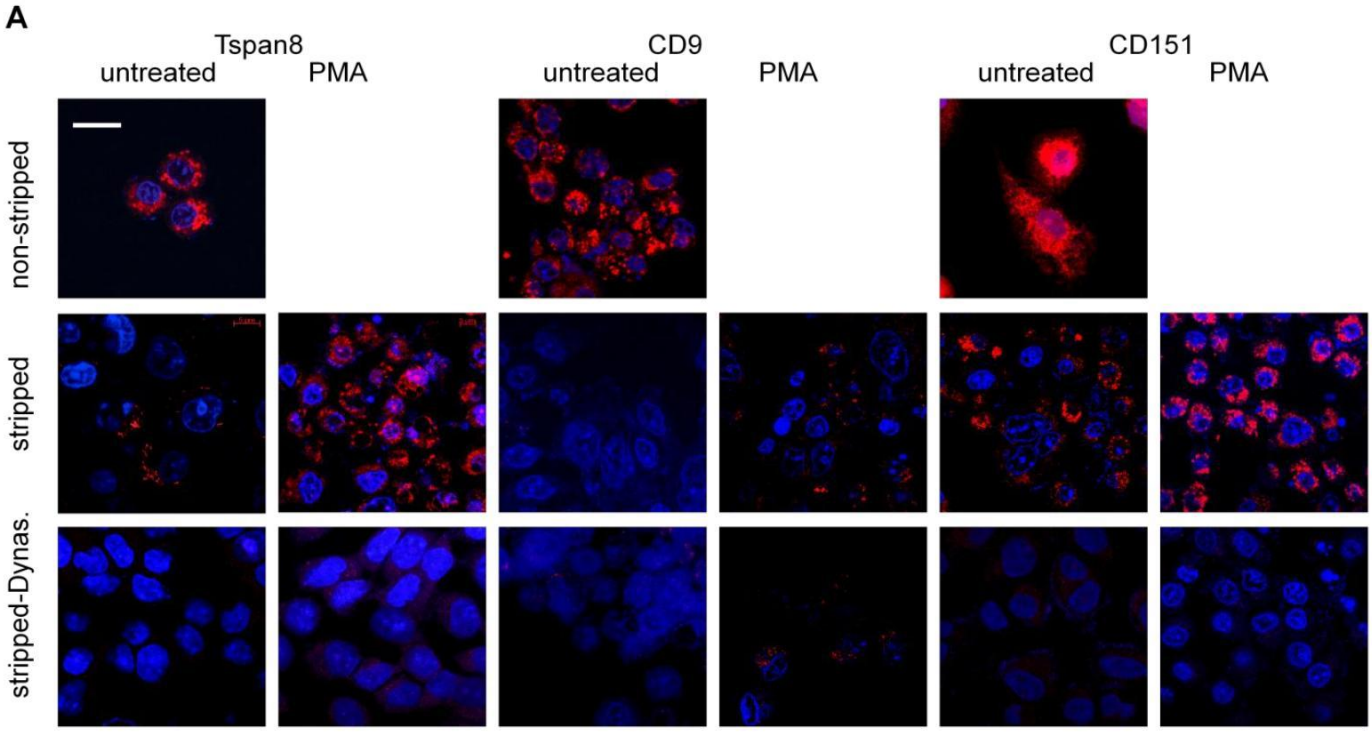


Figure 6. Tspan8 co-localizes and co-internalizes with clathrin: (A and B) Untreated and PMA-treated AS, AS-Tspan8 and AS-Tspan8 chimeras were incubated with anti-CD9, anti-CD151 or D6.1 and were fixed after incubation with / without PMA at 37°C, permeabilized and stained with the secondary dye-labeled antibody and anti-caveolin or anti-clathrin. Overlays of confocal microscopy are shown (scale bar: 10µm).). (C) Lysates of untreated and PMA-treated AS-Tspan8 cells were immunoprecipitated with anti-caveolin or anti-clathrin. Precipitates were separated by SDS-PAGE and after transfer blotted with D6.1, anti-CD9 and anti-CD151. (D) Lysates of untreated and PMA-treated AS-Tspan8 and AS-Tspan8 chimera were immunoprecipitated with anti-clathrin. Precipitates were separated by SDS-PAGE and after transfer blotted with D6.1.

Clathrin-mediated endocytosis requires dynamin, which co-operates with INS2 (Yamabhai et al., 1998). To confirm the involvement of clathrin and INS2 in Tspan8 internalization, AS-Tspan8 cells were PMA-treated in the presence of the dynamin inhibitor Dynasore. Internalization was evaluated by confocal microscopy after stripping surface bound antibody by an acid wash. Internalization of Tspan8, CD151 and TfR, which served as positive control, but not the weak internalization of CD9, was strongly inhibited in the presence of Dynasore (Fig. 7A, B).



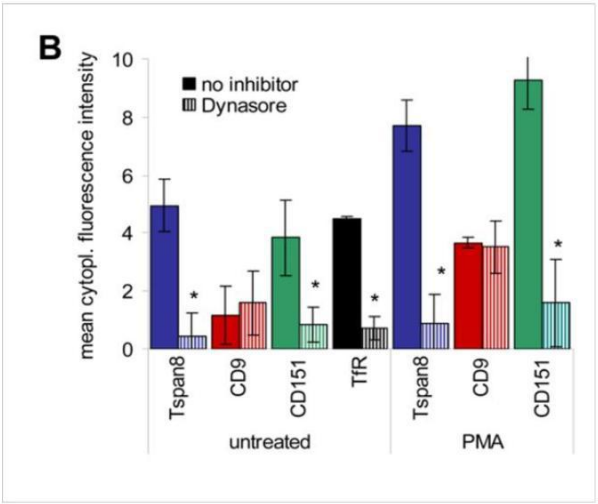


Figure 7. Tspan8-INS2 complex internalization requires dynamin: (A and B) AS-Tspan8 cells were cultured in the presence or absence of Dynasore (80µM) for 5h. Cells were incubated with D6.1, anti-CD9 or anti-CD151 at 4°C, incubated with PMA (30min, 37°C) and transferred on ice. Surface-bound antibody was removed by acid wash (PBS/HCl, pH2.5). Cells were fixed, permeabilized, stained with the secondary dye-labeled antibody and counterstained with DAPI. Internalized Tspan8, CD9 and CD151 were evaluated by confocal microscopy (scale bar: 10µm).). (A) Representative examples. (B)

mean± SD of fluorescence intensity of 10 microscopic fields (corresponding field size as shown in A). Significant inhibition of internalization by Dynasore is indicated by *.

To reinsure the essential contribution of INS2, AS-Tspan8 cells were treated with INS2 siRNA (Fig.8A). When AS-Tspan8-INS2^{kd} cells were stained with D6.1 and thereafter PMA-treated, Tspan8 internalization was strongly reduced, whereas CD151 and weak CD9 internalization was hardly affected (Fig.8B and 8C). Furthermore, co-immunoprecipitation of Tspan8 and dynamin with clathrin was strongly reduced in untreated and was abolished in PMA-treated AS-Tspan8-INS2^{kd} cells (Fig.8D).

Co-localization of Tspan8 with clathrin is in line with the preferential internalization of some tetraspanins via clathrin-coated pits (Helle et al., 2008, Pols et al., 2009, Xu et al., 2009) as well as the direct association of Tspan8 with INS2 (Claas et al., 2005, Claas et al, in prep.).However, there remained the question, why the association of Tspan8 with clathrin and INS2 became strengthened by PMA-treatment, whereas the association with CD9 became weaker. The finding suggests that Tspan8 becomes recruited into membrane domains distinct from TEM (tetraspanin-enriched microdomain) in the resting cell.

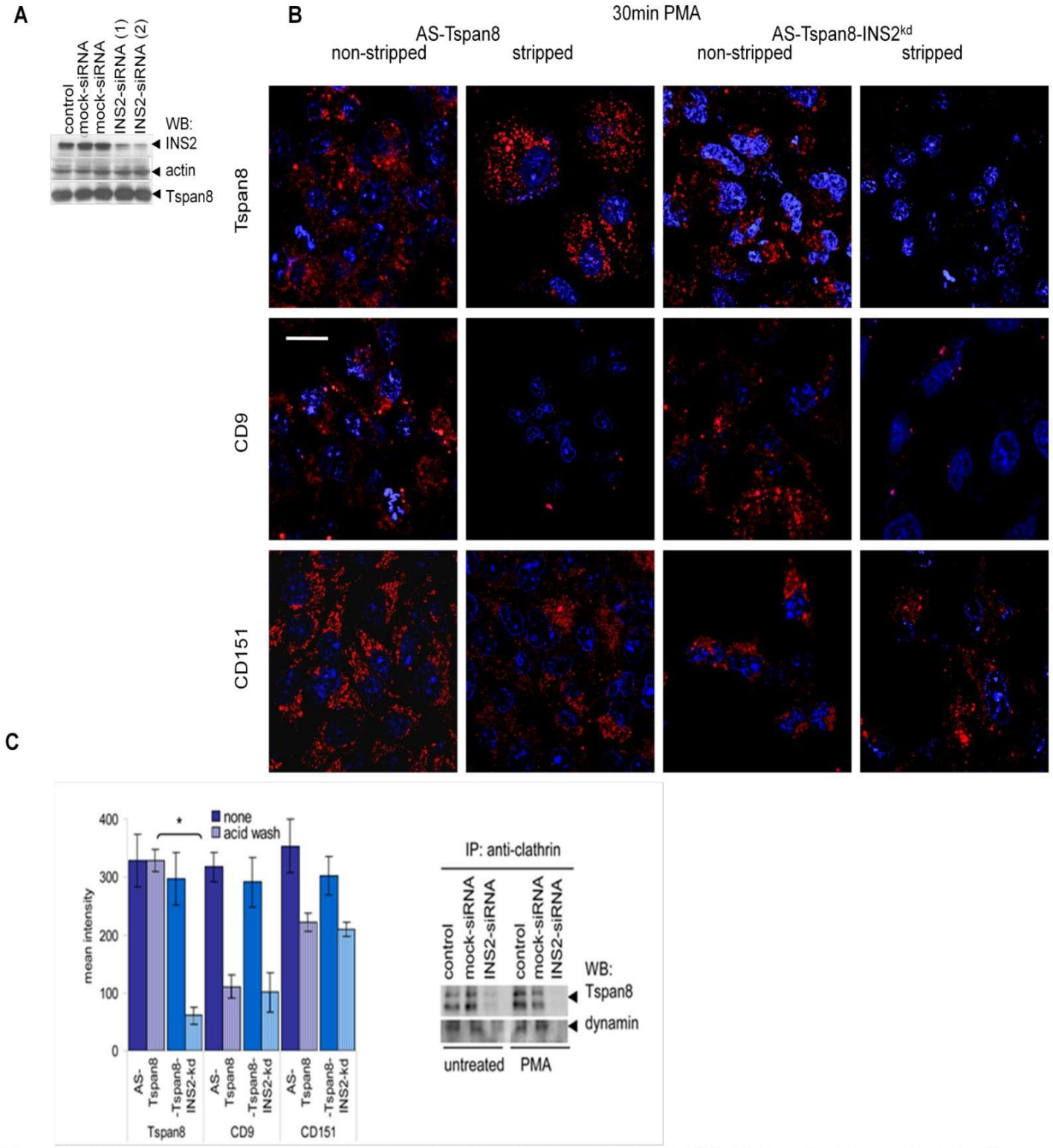


Figure 8. INS2 is a key player in dynamin dependent Tspan8 internalization (A-D) AS-Tspan8 cells were treated with INS2 siRNA. (A) WB of mock- and siRNA-treated AS-Tspan8 cells with D6.1, anti-INS2 and anti-actin. (B and C) AS-Tspan8 and AS-Tspan8-INS2^{kd} cells were incubated with D6.1, anti-CD9 and anti-CD151, incubated with PMA, fixed, permeabilized and stained with the dye-labeled secondary antibody. Internalization was evaluated by confocal microscopy (scale bar: 10µm). (B) Representative examples; (C) Mean± SD of fluorescence intensity of 10 microscopic fields (corresponding field size as shown in D). Significant inhibition of internalization by INS2-siRNA is indicated by *. (D) Lysates of AS-Tspan8 and AS-Tspan8-INS2^{kd} cells were precipitated with anti-clathrin. Precipitates were separated by SDS-PAGE and after transfer blotted with D6.1 and anti-dynamin.

3.4 Changes in Tspan8 partners during internalization

To support the hypothesis of a rearrangement of the Tspan8 web by PMA-treatment, the distribution of Tspan8, CD151 and CD9 in membrane microdomains was evaluated by sucrose density gradient centrifugation. In untreated cells, Tspan8, CD151 and CD9 were enriched in the density fraction of d1.117-d1.130. When cells were treated with MßCD, the tetraspanins shifted towards heavier fractions. After PMA-treatment, Tspan8 and CD151, but not CD9 were enriched in lighter fractions (d1.112-d1.122). Notably, Tspan8 remained in the lighter fractions after MßCD treatment (Fig.9A). Importantly, after PMA-treatment, Tspan8, INS2 and clathrin co-immunoprecipitated in the light fraction of density 1.112 (Fig.9B).

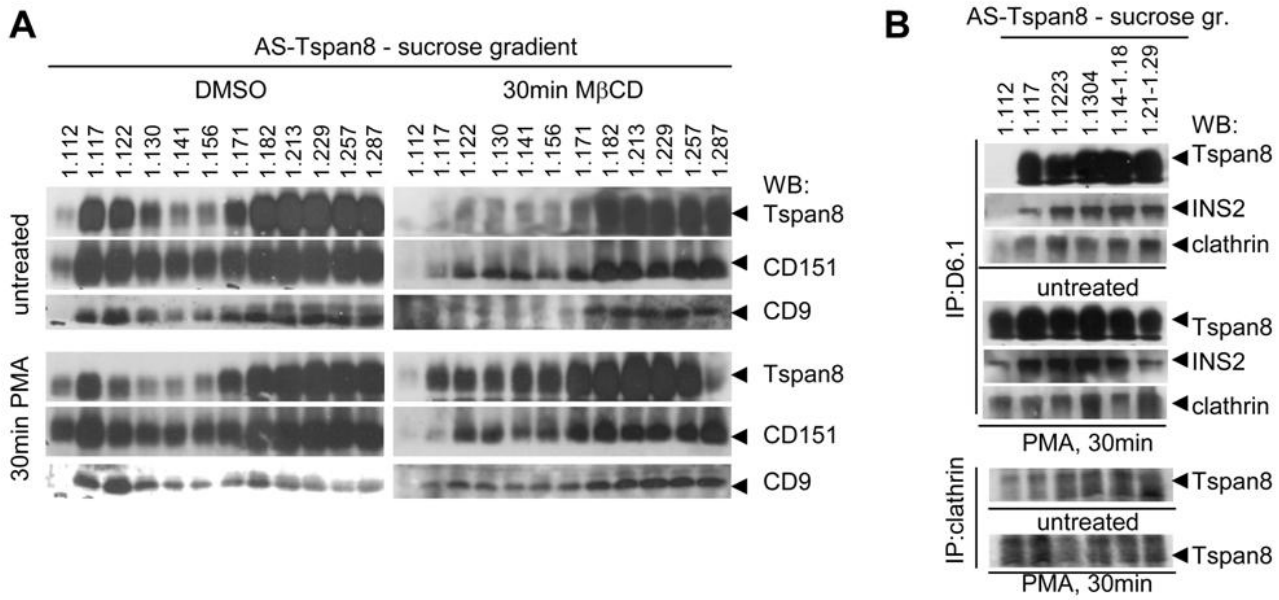


Figure 9. PMA-induced membrane sub-domain redistribution of Tspan8: (A) Untreated and PMA-treated AS-Tspan8 cells were incubated with MβCD for partial cholesterol depletion. Cells were lysed and lysates subjected to sucrose density gradient centrifugation. Fractions were SDS-PAGE separated, transferred and blotted with D6.1, anti-CD9, and anti-CD151. (B) Untreated and PMA-treated AS-Tspan8 cells were lysed and lysates were subjected to sucrose density gradient centrifugation. Fraction 1, 2, 3, 4 and pooled fractions 5-8 and 9-12 were immunoprecipitated with D6.1 or anti-clathrin. Precipitates were separated by SDS-PAGE. Proteins were transferred and membranes were blotted with D6.1, anti-INS2 and anti-clathrin.

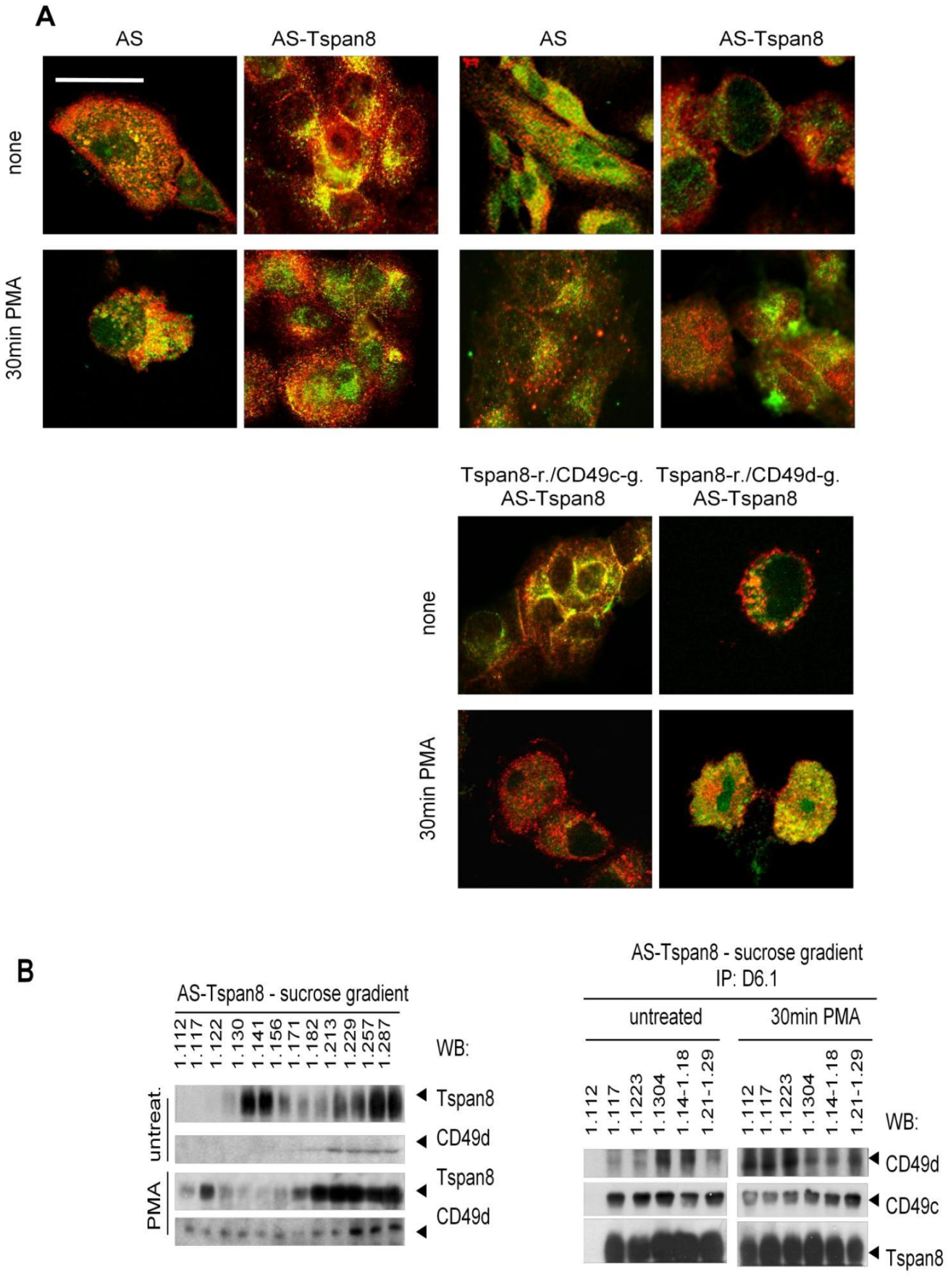
Recovery of the Tspan8-INS2-clathrin complex after PMA-treatment in lighter density fractions supported the hypothesis of a PMA-induced reorganization of Tspan8 complexes, and thus encouraged to search for additional partners that might contribute to reorganization of Tspan8-containing membrane micro-domain. The associations of Tspan8 with integrins vary depending on the integrin profile of the cell. Thus, as shown by previous lab members in another system: in ASML cells, Tspan8 is associated with CD49c and CD104/CD49f (Claas et al., 1998, Herlevsen et al., 2003), the association with CD104 becoming dominant

in PMA-treated cells (Herlevsen et al., 2003, Gesierich et al., 2005). In AS-Tspan8 cells, Tspan8 mostly is associated with CD49c, and weakly with CD49d. After PMA-treatment the association with CD49c becomes weak and is restricted to the cell membrane, whereas cytoplasmic Tspan8 strongly associates with CD49d. This also accounts for co-immunoprecipitation. Only after PMA-treatment, CD49d is recovered in the light density fractions, where it co-immunoprecipitates with Tspan8. On the other hand, co-immunoprecipitation of CD49c with Tspan8 becomes weaker and is mostly restricted to the heavier density fractions (Fig.10A, 10B).

Exchange of the CD151 or CD9 C-terminal region does not affect the association of Tspan8 with CD49d during PMA-induced internalization. On the contrary, exchange with the CD151 and, far more pronounced, the CD9 N-terminal region interferes with the co-localization of Tspan8 and CD49d (Fig.10C). Co-immunoprecipitation of CD49c and CD49d with Tspan8 chimeras confirms that the association with CD49d becomes strengthened by PMA-treatment, but is significantly weakened, when the N-terminal region of Tspan8 is exchanged by that of CD9. In contrast, CD49c readily co-immunoprecipitated with Tspan8/CD9n and CD9c+n after PMA-treatment (Fig.10D).

As CD49d associates with Tspan8 during PMA-treatment / internalization and co-immunoprecipitates with Tspan8 mostly in the light density fractions, CD49d could be a potential candidate for the recruitment of Tspan8 towards INS2 and clathrin. Obviously, CD49d remains Tspan8-associated during the vesicular transport, as CD49d co-immunoprecipitates with Tspan8 in exosomes which we also have shown before (Nazarenko et al., 2010).

If our hypothesis is correct that Tspan8 by its association with INS2 and the recruitment of CD49d becomes enriched in MVB such that the complex is exocytosed rather than degraded or reintegrated in the plasma membrane, PMA stimulation of AS-Tspan8 cells should be accompanied by reduced matrix and cell adhesion.


A
AS
AS-Tspan8
AS
AS-Tspan8
none
30min PMA
Tspan8-r./CD49c-g.
AS-Tspan8
Tspan8-r./CD49d-g.
AS-Tspan8
none
30min PMA
B
AS-Tspan8 - sucrose gradient
1.112
1.117
1.122
1.130
1.141
1.156
1.171
1.182
1.213
1.229
1.257
1.287
WB:
untreat.
PMA
Tspan8
CD49d
Tspan8
CD49d
AS-Tspan8 - sucrose gradient
IP: D6.1
untreated
30min PMA
1.112
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1.1223
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1.21-1.29
WB:
CD49d
CD49c
Tspan8

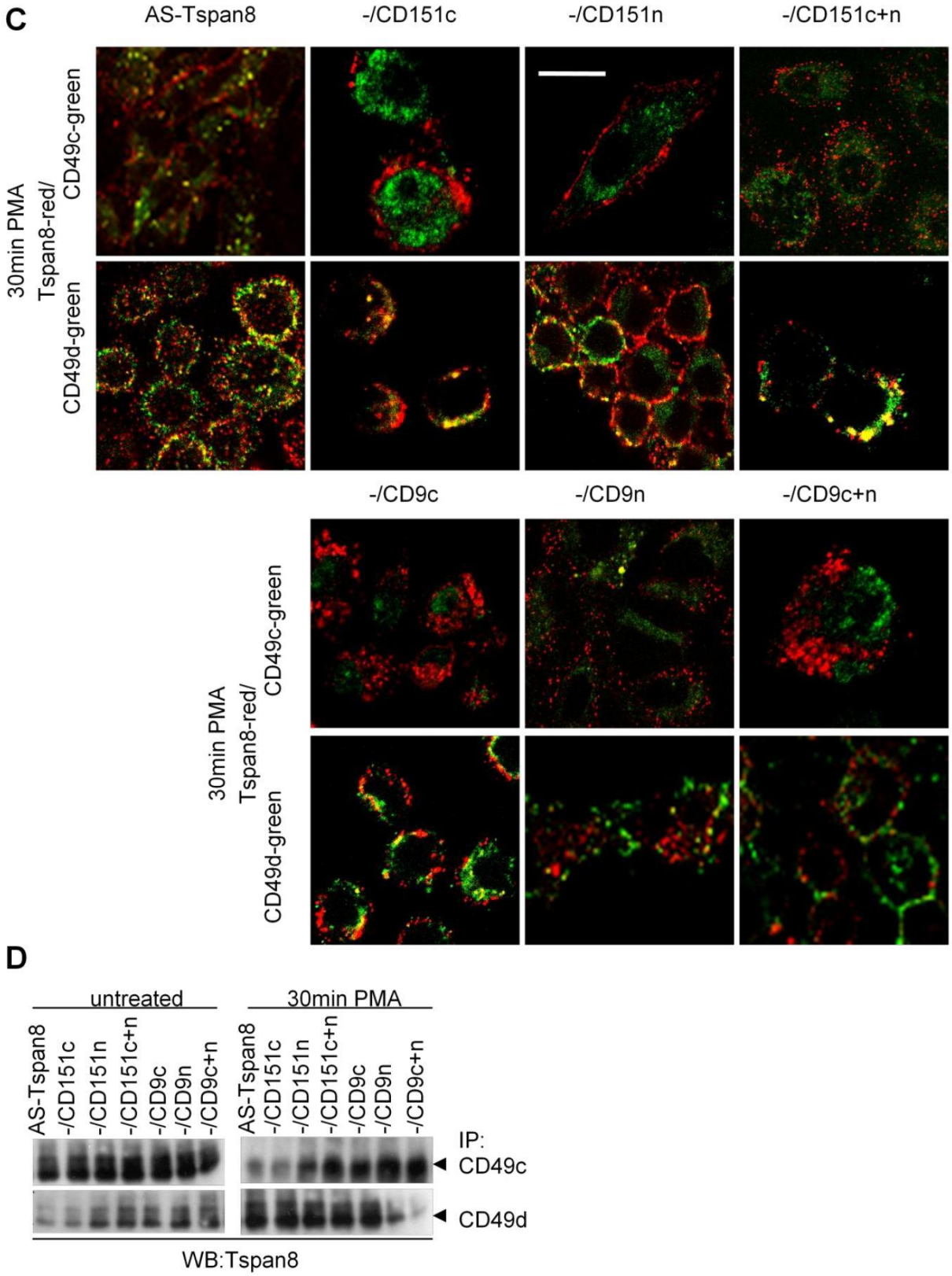


Figure 10. **During PMA-induced internalization, Tspan8 associates with CD49d**: (A) Untreated and PMA-treated AS and AS-Tspan8 cells were stained with anti-CD9 or D6.1 and anti-CD49c or anti-CD49d. Overlays of confocal microscopy are shown (scale bar: 10µm). (B) Sucrose density gradient fractions of lysates of untreated and PMA-treated AS-Tpan8 cells were blotted with D6.1 and anti-CD49d or were precipitated with D6.1. Precipitates were separated by SDS-PAGE and after transfer blotted with D6.1, anti-CD49c and anti-CD49d. (C) PMA-treated AS-Tspan8 chimeras were stained with D6.1 and anti-CD49c or anti-CD49d. Overlays of confocal microscopy are shown (scale bar: 10µm).

(D) Lysates of untreated and PMA-treated AS-Tspan8 and AS-Tspan8 chimera were immunoprecipitated with anti-CD49c or -CD49d. Precipitates were separated by SDS-PAGE and after transfer blotted with D6.1.

3.5 Adhesiveness and motility of PMA-stimulated AS-Tspan8 cells

Matrix adhesion, cell-cell adhesion and migration have been evaluated with AS, AS-Tspan8 and all AS-Tspan-chimeras. As the most striking differences were observed upon exchange of the Tspan8 N-terminal region by that of CD9, only the impact on functional activity of the latter chimera in comparison to AS and AS-Tspan8 cells will be presented.

Unstimulated AS and AS-Tspan8 cells readily adhere to FN and Ln5, but poorly to BSA. PMA stimulation decreased adhesiveness of AS-Tspan8 to FN and Ln5, but adhesion of AS-Tspan8/CD9n cells was not affected (Fig.11A). D6.1 significantly inhibited Ln5 adhesion of AS-Tspan8. PMA-treatment strongly reduced inhibition by D6.1. Instead, weak inhibition of AS-Tspan8/CD9n was not affected by PMA-treatment. The very same feature of inhibition was seen with anti-CD49d. Instead, anti-CD49c equally well inhibited untreated and PMA-treated AS-Tspan8 cells and more efficiently inhibited adhesion of PMA-treated than untreated AS- Tspan8/CD9n cells (Fig.11B). From there, we conclude that Tspan8 contributes to matrix adhesion only in the resting state, where Tspan8 is preferentially associated with CD49c.

We observed recently that the AS-Tspan8 cells adhered more readily to RAEC than AS cells (Nazarenko et al., 2010). Adhesion to RAEC was strongly reduced in AS-Tspan/CD9n cells. PMA-treatment only reduced adhesion of AS-Tspan8 cells (Fig.11C). Similar to matrix adhesion, D6.1 and anti-CD49d inhibited RAEC binding significantly less efficiently when AS-Tspan8 cells were PMA-treated.

The weaker inhibition of AS-Tspan8 binding to RAEC by anti-CD49c was not affected by PMA-treatment. Weak inhibition of AS-Tspan8/CD9n binding to RAEC by D6.1, anti-CD49d and anti-CD49c was not affected by PMA-treatment (Fig.11D). Different from adhesion, PMA-treatment strengthened migration. Wound closure of AS-Tspan8 cells proceeded faster than that of AS cells and AS-Tspan8/CD9n cells and was further accelerated in the presence of PMA.

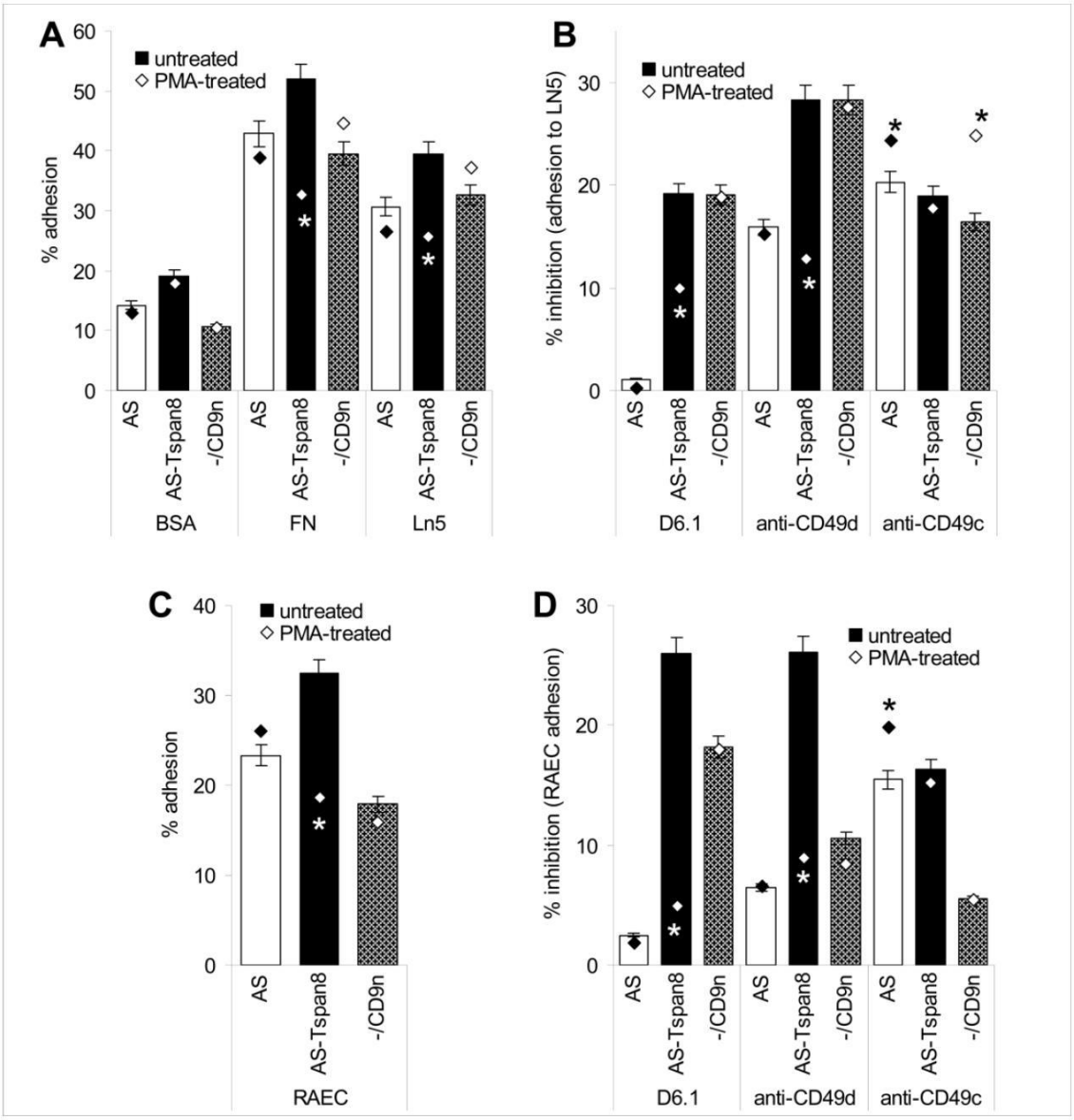


Figure 11. Impact of the N-terminal regions of Tspan8 on matrix and cell adhesion: (A) Adhesion to Ln5 and FN of untreated and PMA-treated AS, AS-Tspan8 and AS-Tspan8/CD9n cells. The percentage of adherent cells was evaluated after 2h incubation at 37°C. (B) Untreated and PMA-treated cells as in (A) were seeded on Ln5 in the presence of D6.1, anti-CD49c and anti-CD49d. The percent inhibition as compared to (A) is shown. (C and D) CFSE-labeled untreated and PMA-treated AS, AS-Tspan8 and AS-Tspan8/CD9n cells were seeded on a monolayer of RAEC and incubated for 2h. The percentage of adherent cells is shown. (D) Adhesion to RAEC was evaluated in the presence of D6.1, anti-CD49c or anti-CD49d. The percent inhibition as compared to cells incubated in the presence of control IgG is shown. In A-D mean values± SD of triplicates are presented; significant ($p<0.01$) PMA-induced differences are indicated by *.

However, after 48h PMA-treatment, all 3 lines had closed or nearly closed the wound. D6.1 and anti-CD49d only inhibited migration of AS-Tpan8 cells. On the contrary, anti-CD49c most efficiently inhibited migration of AS and AS-Tpan8/CD9n cells (Fig.12A, B). AS, AS-Tspan8 and AS-Tspan8/CD9n cells did not differ significantly in trans-well migration. However, only the migratory activity of AS-Tspan8 cells was strongly increased in the presence of PMA (Fig.12C). Importantly, cable formation on matrigel was completely prevented in AS-Tspan8/CD9n and AS-Tspan8/CD9c+n chimeras and was weakened in AS-

Tspan8/CD151n chimeras as compared to AS-Tspan8 wild type cells. This accounted for the number of cable-forming cells as well as for the cable length (Fig.13A, B).

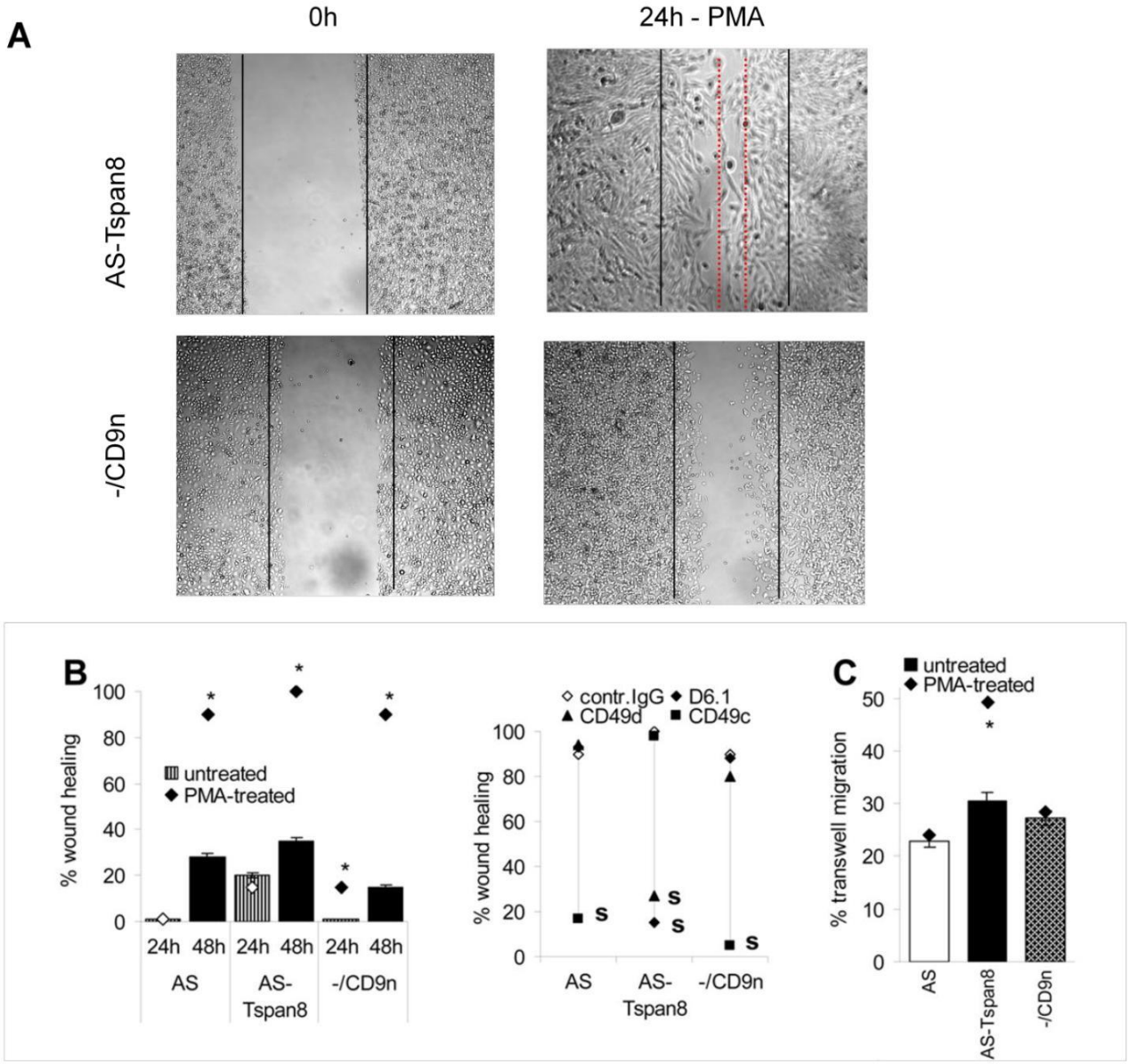


Figure 12. Impact of the Tspan8 N-terminal region on cell migration: Migration of untreated and PMA-treated AS, AS-Tspan8 and AS-Tspan8/CD9n cells in a wound healing assay was evaluated after 24h and 48h by light microscopy. The impact of D6.1, anti-CD49c and anti-CD49d on wound healing was evaluated in PMA-treated cells after 48h. (A) Representative examples of images of wound healing in AS-Tspan8 and AS-Tspan8/CD9n cells. Black lines represent the initial scratch and the red dotted lines denote the migrated front. (B) The mean percentage of wound closure in dependence of the presence of PMA and antibodies of three independent experiments. (C) Mean values± SD of transwell migration of CFSE- labeled untreated and PMA-treated AS, AS-Tspan8 and AS-Tspan8/CD9n cells. (B & C) Significant differences ($p<0.01$) in dependence on PMA-treatment are indicated by * and in dependence on the presence of antibodies by **s**.

Thus, AS-Tspan8 cells responded to PMA-treatment with pronounced migration and reduced adhesiveness. Both activities were mitigated, when the N-terminal region was exchanged by that of CD9.

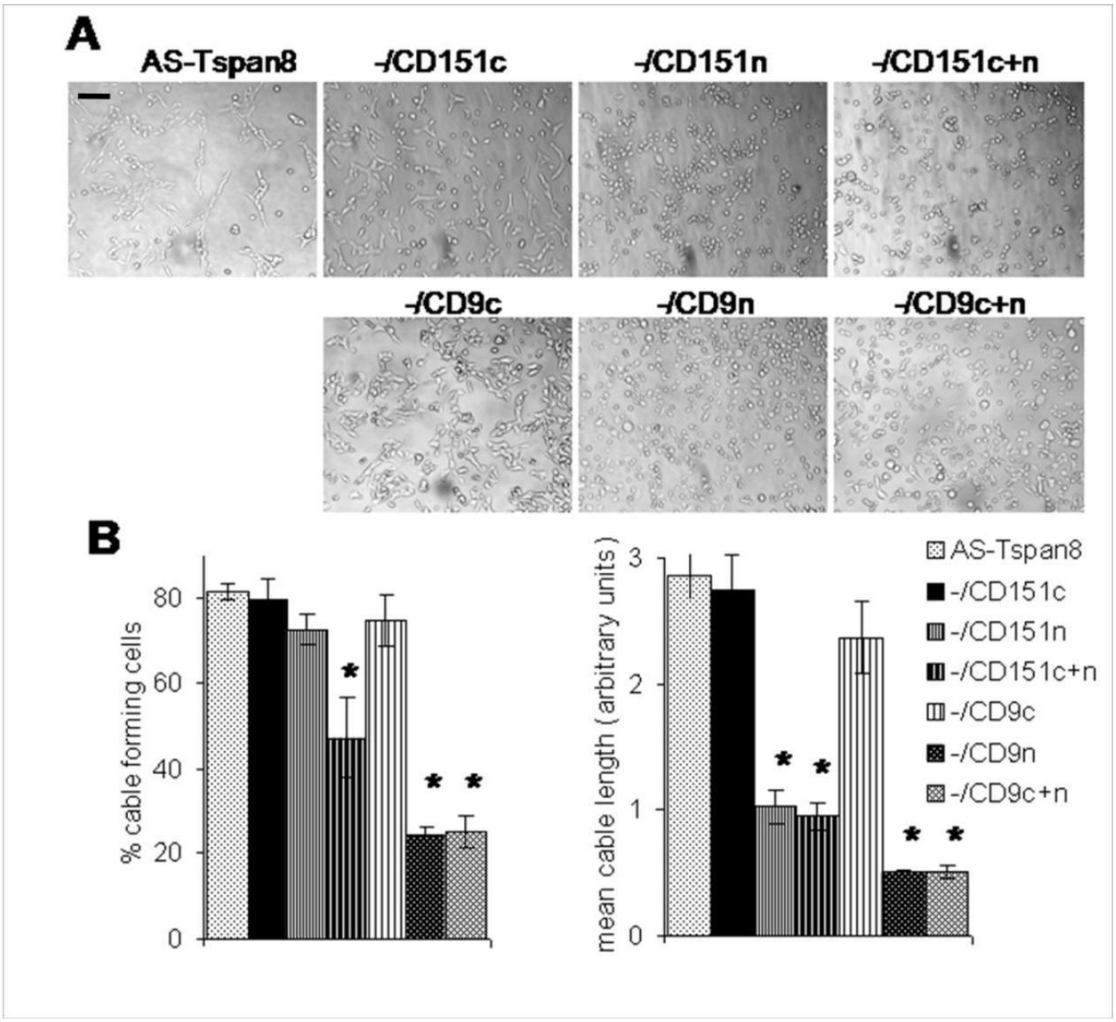


Figure13. Impact of the Tspan8 N-terminal region on cable formation. AS-Tspan8 and AS-Tspan8-chimeras were seeded on matrigel. Cable formation and sprouting was evaluated after 24h by light microscopy. (A) Representative examples (scale bar: 20µm); (B) the mean numbers of cells forming cables and the relative mean± SD of cable length in 10 independent areas. Significant differences ($p<0.01$) compared to AS-Tspan8 cells are indicated by *.

Taken together, Tspan8 takes a selective route of internalization due to its association with INS2 and the recruitment of CD49d in the internalization complex. This selective internalization could well account for the functional activity of AS-Tspan8 exosomes provided the Tspan8 internalization complex is recovered in exosomes and contributes to target cell selection. Therefore, I started to explore the engagement of this internalization in exosome assembly and target cell selection.

3.6 Subpopulations within the AS-Tspan8 derived exosomes

Since we had seen marked differences in the internalization of Tspan8 as compared to CD9 and CD151, and the CD9-CD151-Tspan8 complex detected in the cell membrane of resting cells was not recovered after internalization, it seemed plausible that these differences may lead to sub-populations of exosomes deriving from the same cell, which differ in Tspan8 and CD9 content. To sort this, we incubated AS-Tspan8 and the AS-Tspan8-CD9n - derived exosomes with D6.1 or anti-CD9-coated latex beads. Tetraspanin expression of latex-bead-bound positively selected population as well as non-bound exosome populations (after binding to nascent, non-coated latex beads) was evaluated by flow cytometry after incubations with D6.1 or anti-CD9 and with APC- conjugated secondary antibody.

These, however, could not be verified. Exosomes binding to D6.1-coated beads were both-Tspan8 and CD9 positive and exosomes that did not bind to D6.1-coated beads were mostly CD9- and Tspan8- negative. Instead, anti-CD9-coated beads did not capture all exosomes. Furthermore, less $Tspan8^+$ than $CD9^+$ exosomes were bound and more $Tspan8^+$ than $CD9^+$ exosomes were recovered in the non-binding fraction (Fig.14).

From these findings we concluded that during the process of formation of MVB, differences in the internalization of individual tetraspanins become, at least partly, hidden, which may be facilitated by the continuity of the process, such that distinctly internalized endosomes and endosomes derived from the synthetic pathway are concomitantly recovered after the collection period of 48h. Nonetheless, not all Tspan8 expressing exosomes could be captured by CD9-coated beads.

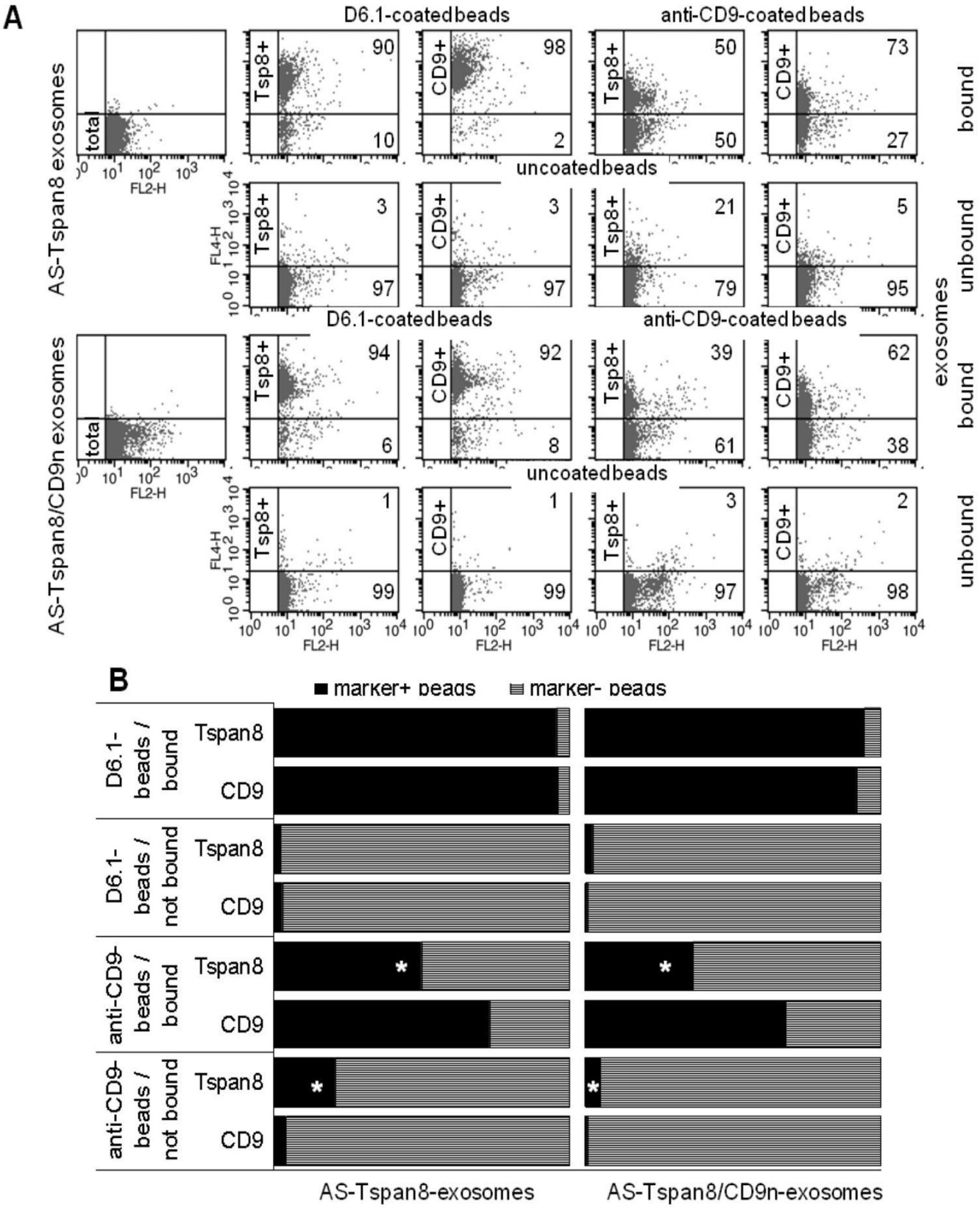


Figure 14. Tetraspanin recovery in exosomes. DHPE-labeled, AS-Tspan8- and AS-Tspan8/CD9n- exosomes were incubated with D6.1- or anti-CD9-coated latex beads. Bound aa well as unbound exosomes (which were then loaded on native/ uncoated latex beads) were stained with D6.1 or anti-CD9 and counterstained with anti-mIgG-APC. (A) Examples; (B) Mean recovery in 3 separate experiments. Significant differences in binding of $Tspan8^+$ and $CD9^+$ exosomes are indicated by *.

3.7 Differences in the tetraspanins webs

In view of the latter finding, the existence of well- defined subpopulations of exosomes with respect to content of CD9 and Tspan8 seemed unlikely. On the other hand, there are remarkable differences in effects of AS, AS-Tspan8, ASML and AS-Tspan8-CD104 exosomes on EC, where only AS-Tspan8 exosomes could activate ECs, as shown by others in our group (Gesierich et al., 2006; Nazarenko et al., 2010). Thus, we speculated that different tetraspanin-complexes on the exosomes account for the differences in target selection.

I outlined above that Tspan8, distinct to CD9 and CD151, during the internalization process associates with CD49d, while, similar to CD9 and CD151, being preferentially associated with CD49c in the membrane of the resting cell. To find out if these differences among the tetraspanin-complexes generated during internalization were maintained in exosomes, we carried out immunoprecipitations against Tspan8, CD9, CD151 and CD81 on biotinylated and non-biotinylated AS-, AS-Tspan8-, AS-Tspan8/CD9n- and AS-Tspan8-CD104-exosomes. After 10% SDS PAGE, western blotting with streptavidin-conjugated horse radish peroxidase (Strep-HRP) was performed with IPs done on biotinylated exosomes (Fig.15) and, silver staining was carried out with non-biotinylated samples to have an overview of differences in tetraspanin webs, if any.

In fact, these differences in the tetraspanin-complexes generated during the internalization process were maintained in exosomes. Anti-CD49c precipitated Tspan8, CD9 and CD151 in AS-, AS-Tspan8-, AS-Tspan8/CD9n- and AS-Tspan8-CD104-exosomes. Instead, anti-CD49d precipitated only Tspan8 and CD151 in AS-Tspan8-exosomes and Tspan8 in AS-Tspan8-CD104 exosomes. B5.5 (anti-CD49f/CD104) also precipitated Tspan8 in AS-Tspan8-CD104 exosomes (Fig.15A and 15B).

As revealed by immunoprecipitations with anti-CD151, -CD9, -CD81 and -Tspan8 (D6.1), at least one additional protein was selectively precipitated by D6.1 and only in case of AS-Tspan8 exosomes (Fig.15C and 15D). This and additional proteins selectively recovered in

the exosomes of AS, AS-Tspan8, AS-Tspan8/CD9n and AS-Tspan8-CD104 are currently being identified with MALDI- TOF analysis.

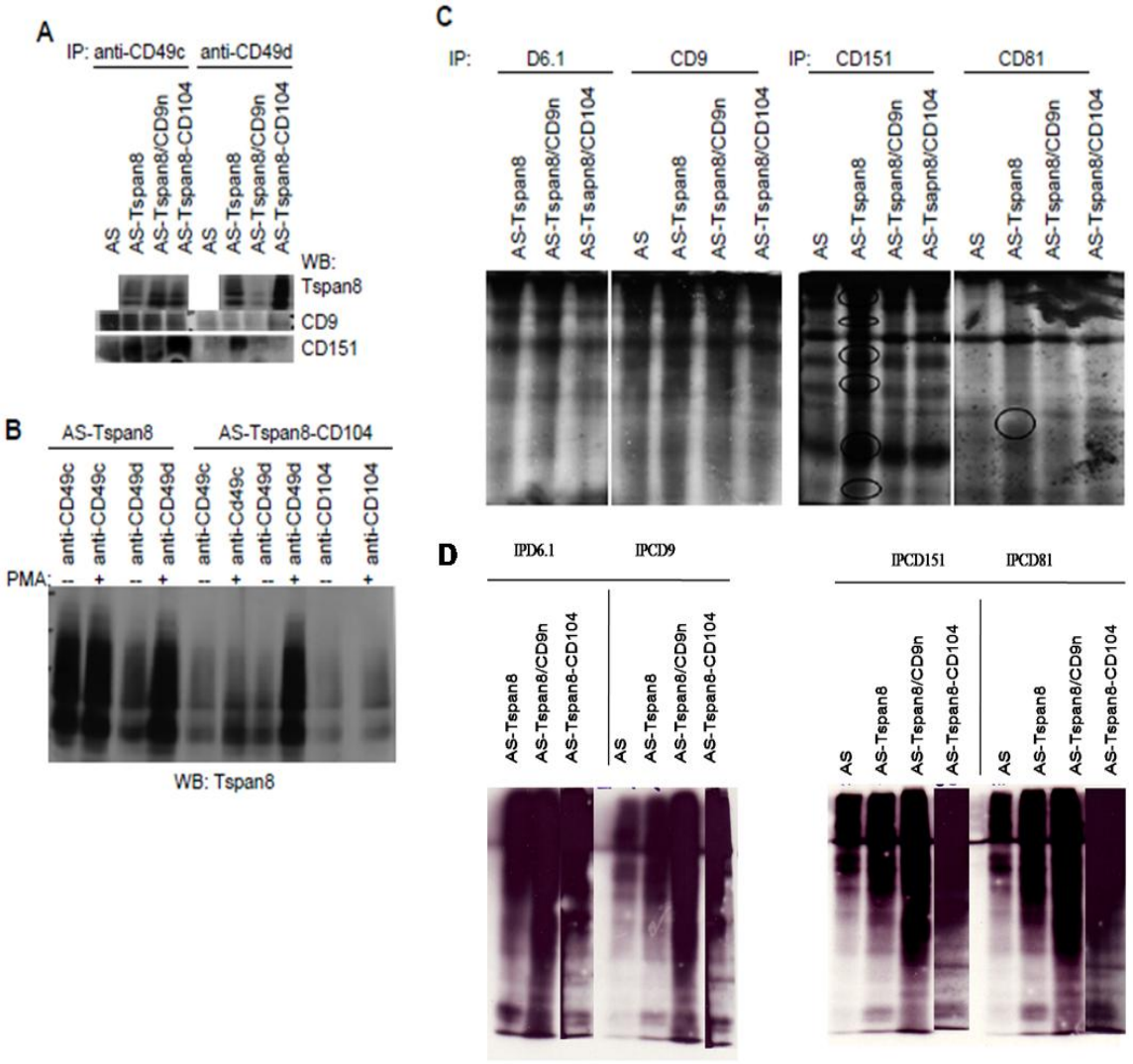
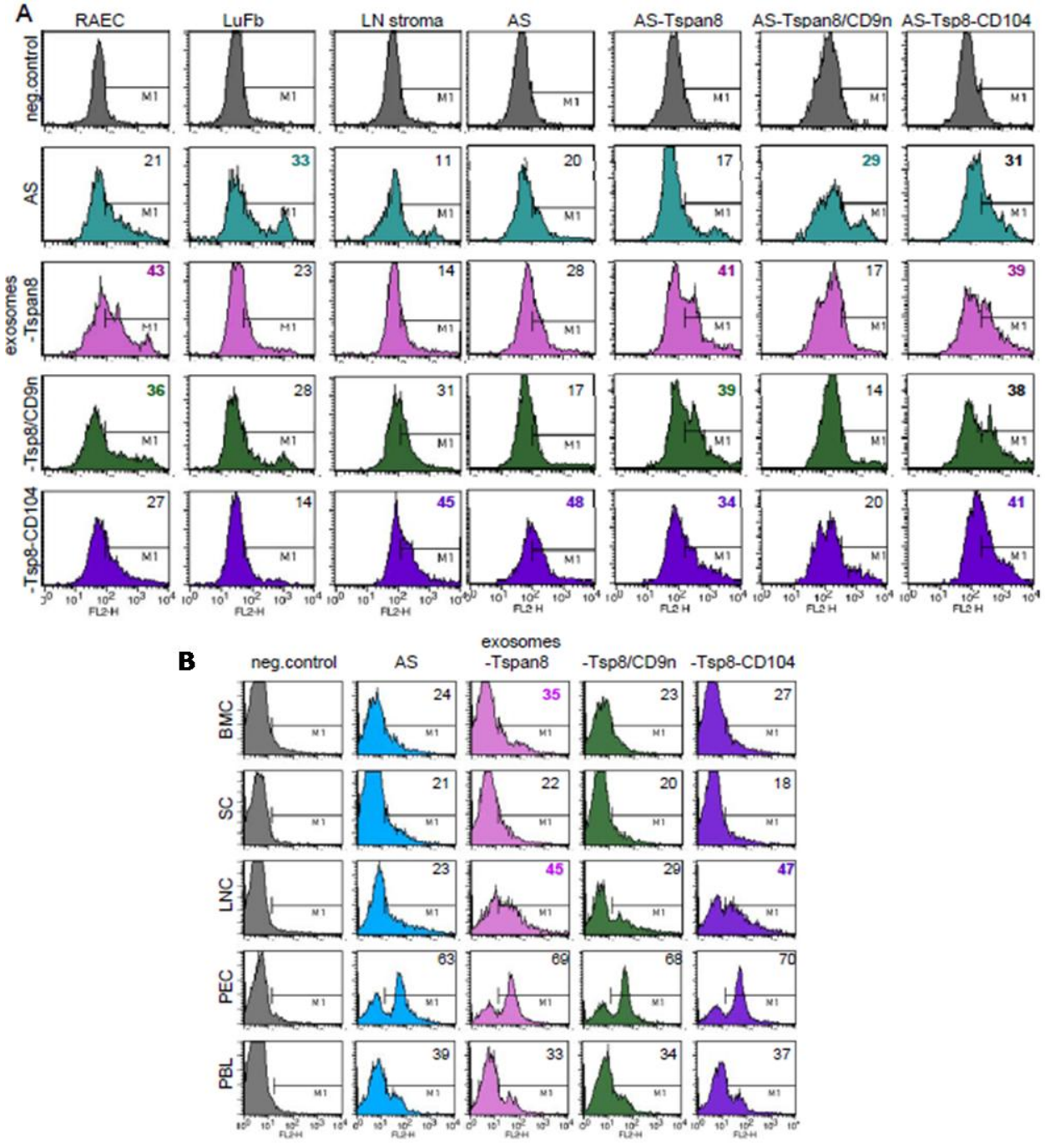


Figure 15. Recovery of tetraspanin complexes in exosomes: (A and B) AS, AS-Tspan8, AS-Tspan8/CD9n and AS-Tspan8-CD104 exosomes were lysed with Brij96 and precipitated with anti-CD49c, anti-CD49d and B5.5 (anti-CD49f/CD104). After SDS-PAGE separation and transfer, membranes were blotted with D6.1, anti-CD9 and anti-CD151. Where indicated (B), exosomes were collected from PMA-stimulated AS-Tspan8 and AS-Tspan8-CD104 cells. (C) Exosomes from the cell lines indicated above were immunoprecipitated with D6.1, anti-CD9, anti-CD151 and anti-CD81. Immunoprecipitates were separated by 10% SDS-PAGE and silver stained. The indicated bands were excised and analyzed by MALDI-TOF. (D) Immunoprecipitation with lysates of biotinylated exosomes samples done as in (C), 10% SDS-PAGE was run, followed by transfer to nitrocellulose membrane and blotting with streptavidin-HRP to get an overview of tetraspanin complexes.

These findings confirmed that the exosomal tetraspanin web differs in dependence on the presence of Tspan8. In addition, Tspan8 web recruitment into exosomes also becomes affected by the available integrins.

3.8 Exosome binding to cells.

Exosomes derived from AS, AS-Tspan8 and AS-Tspan8-CD104 have been shown to differ functionally (Gesierich, 2006; Nazarenko et al., 2010), for e.g., AS-Tspan8 as compared to AS exosomes preferentially bound to and are taken up by endothelial cells (Nazarenko et al., 2010). In addition, differences were seen in the tetraspanin complexes of the AS, AS-Tspan8, AS-Tspan8-CD9n and AS-Tspan8-CD104 derived exosomes. Thus, it became likely that the tetraspanin complexes on exosomes contribute to target cell selection. For this, it was imperative to first check whether exosomes indeed bind to different cells and tissues *in vitro*.



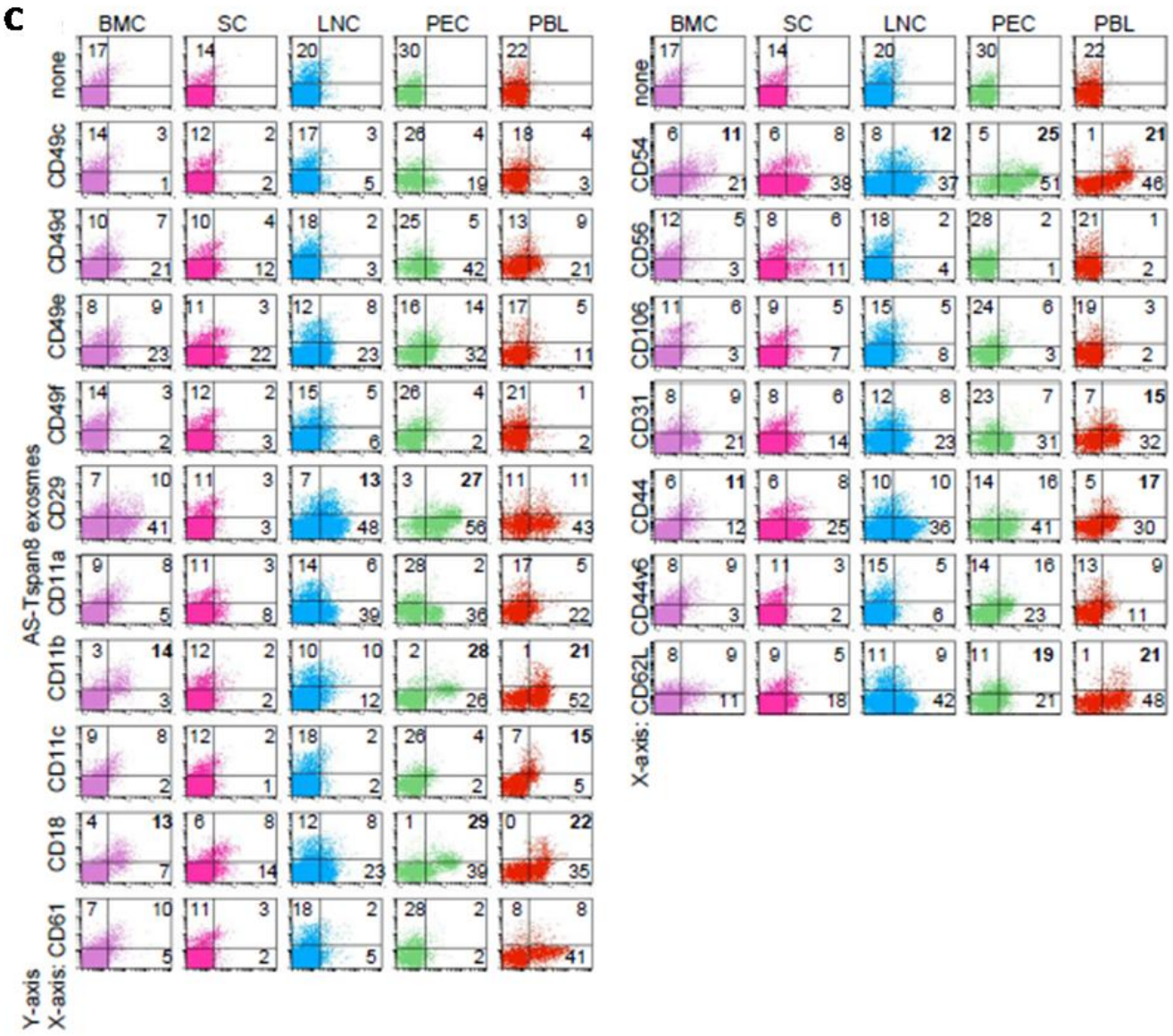


Figure16. ***In vitro*** **binding and uptake of exosomes by cell lines and hematopoietic cells.** DHPE-labeled AS, AS-Tspan8-, AS-Tspan8/CD9n- and AS-Tpan8-CD104-exosomes were incubated with (A) RAEC, LuFb, LnStr, AS, AS-Tspan8, AS-Tspan8/CD9n and AS-Tspan8-CD104 cells or (B and C) with freshly harvested BMC, SC, LNC, PEC and PBL. Exosome uptake was evaluated by flow cytometry. (C) Samples as in B were counterstained by the indicated antibodies. Exosome binding / uptake on marker$^+$ cells was only evaluated for AS-Tspan8 exosomes. Cells in upper left quadrants have taken up exosomes or, are exosome$^+$, cells in the lower right quadrant are marker$^+$, and in the upper right quadrant represents cells that are marker$^+$ and have taken up exosomes.

When dye-labeled AS-, AS-Tspan8-, AS-Tspan8/CD9n- and AS-Tspan8-CD104 exosomes (20µg/ml) were incubated with endothelial cells (RAEC), lung fibroblasts (LuFb), lymph node stroma cells (LNstr) and the tumor lines AS, AS-Tspan8, AS-Tspan8/CD9n and AS-Tspan8-CD104 exosome binding clearly differed depending on the exosome donor line and the target cell. Thus, AS-Tspan8- and AS-Tspan8/CD9n-exosomes were preferentially taken up by endothelial cells as well as by AS-Tspan8 and AS-Tspan8-CD104 tumor cells. Instead,

AS-exosomes preferentially bind LuFb, AS-Tspan8/CD9n and AS-Tspan8-CD104 cells. Distinctly, too, AS-Tspan8-CD104 exosomes strongly bind LNStr, AS and AS-Tspan8-CD104 (Fig.16A). Similar studies were performed on freshly harvested hematopoietic cells (Fig16B). In vitro exosomes uptake by hematopoietic cells again differed with the source of the exosomes. BMC and LNC more readily took up AS-Tspan8-exosomes and AS-Tspan8-CD104 exosomes also bound more strongly to LNC. As shown for AS-Tspan8 exosomes, these exosomes were preferentially taken up by $CD11b^+/CD18^+$ as well as by $CD54^+$ cells. Exosome uptake by e.g. $CD11c^+$, $CD31^+$, $CD44^+$ and $CD62L^+$ cells was restricted to the origin of the hematopoietic cells and was mostly seen in PBL (Fig.16C).

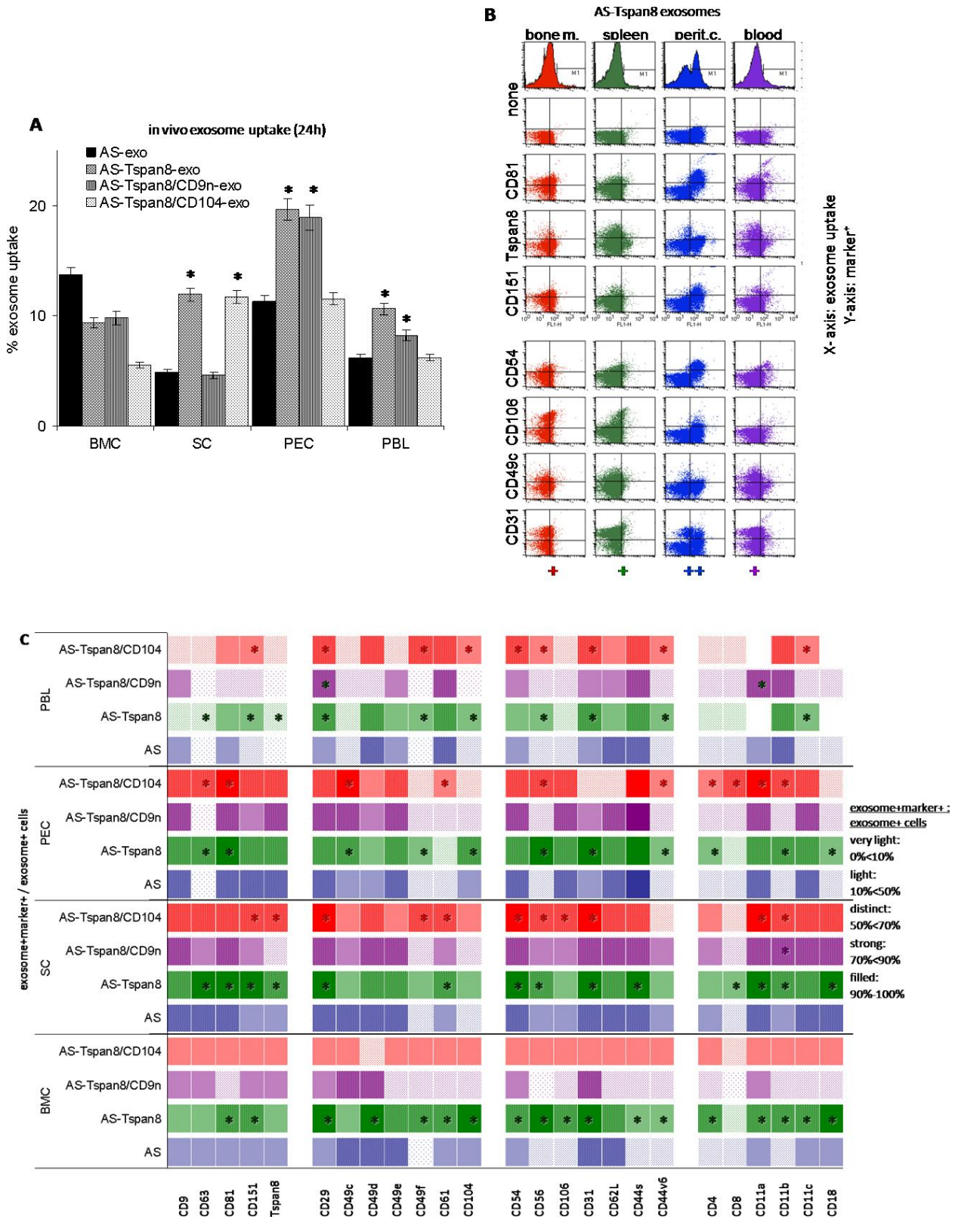
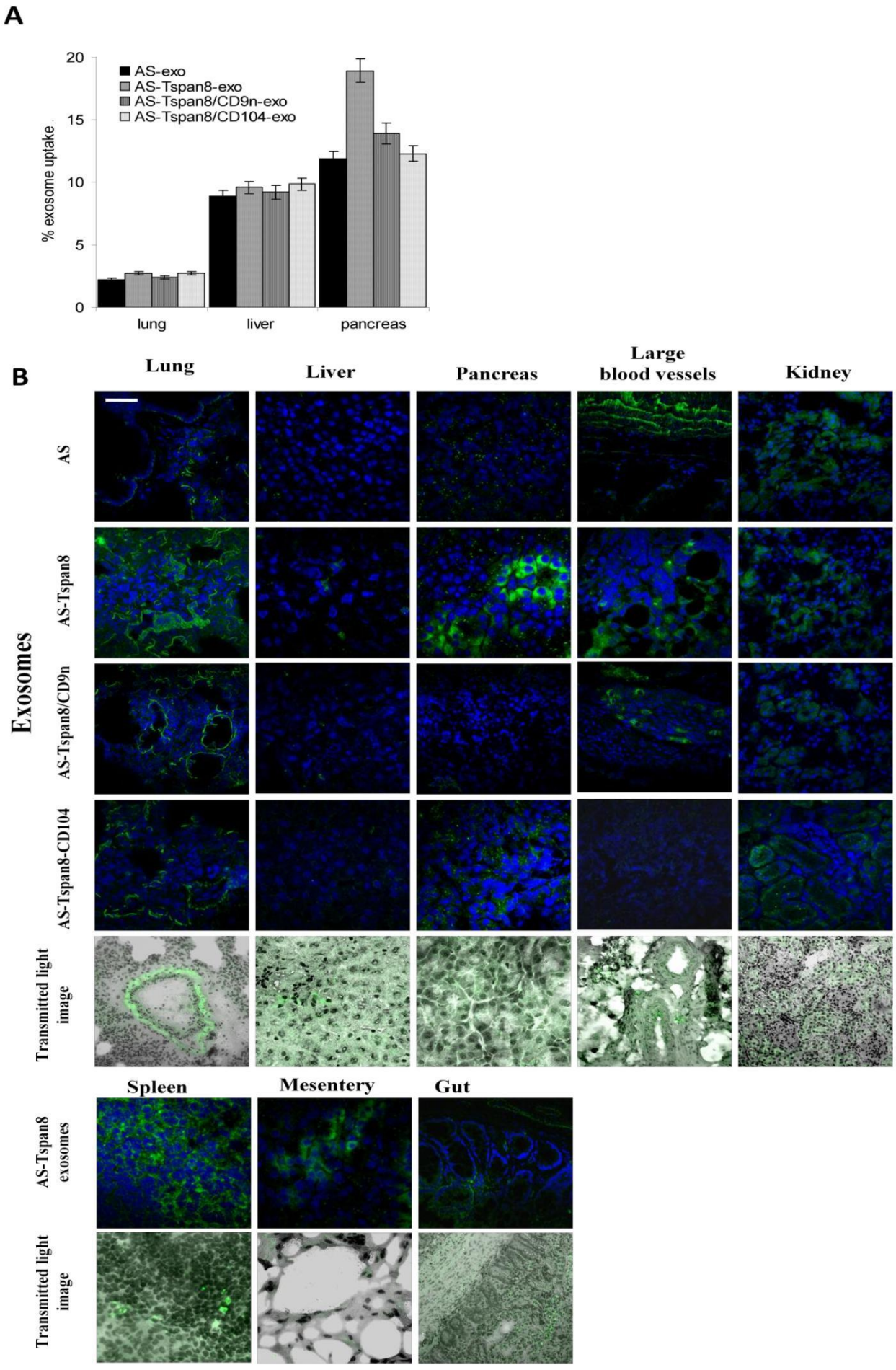


Figure 17. ***In-vivo*** **uptake of exosomes.** 250ug of SP-Dio$_{18}$ (3) labeled AS, AS-Tspan8, AS-Tspan8/CD9n or AS-Tspan8-CD104-exosomes were injected i.v. in the tails of 9-11week old BDX rats. 24h after injection, rats were sacrificed, spleen cells (SC) were prepared from mincing spleen, peripheral blood mononuclear cells were obtained by Ficoll-Hypaque centrifugation gradient of heparinized blood, and peripheral exudate cells (PEC) were collected after washing the peritoneal cavity with PBS-heparin. These cells were then subjected to FACS to look for exosome signals. (A) Percentage of BMC, SC, PEC and PBL that had taken up exosomes are shown (mean± SD of 4 rats, significant differences are indicated by *). (B and C) Cells were counterstained with antibodies for the indicated markers. (B) Representative example (flow cytometry): cells in upper left quadrant are marker^{+}, cells in the lower right quadrant are

exosome$^+$, and cells in the upper right quadrant took up exosomes and are marker$^+$; (C) Mean values of the percentage of double exosome$^+$/ marker$^+$ cells/ exosome$^+$ cells in the indicated organs. The percentages of exosome$^+$marker$^+$cells were grouped in negative (0-10%), weak co-staining (10<50%), distinct co-staining (50<70%), strong co-staining (70-90%) and (nearly) complete co-staining(90<100%). A significant increase in exosome$^+$/ marker$^+$ versus exosome$^+$ marker$^-$ cells in comparison to AS exosomes is indicated by *****.

Particularly interesting was the question, especially on seeing the differences in the *in vitro* studies, whether exosomes will be taken up in vivo or will be captured after intravenous application in spleen and liver and become degraded. For this, 250µg of SP-Dio$_{18}$ (3) labeled AS, AS-Tspan8, AS-Tspan8/CD9n, and AS-Tspan8-CD104 exosomes were injected intravenously in the tail of 9-11wk old BDX rats. 24h later, the animals were sacrificed and blood, lymph nodes, thymus, spleen, pancreas, lung, liver, and bone marrow were excised. Peripheral blood leukocytes (PBL) were obtained via Ficoll-Hypaque gradient centrifugation. Parts of organs were frozen for immunohistological studies. Single cell suspension from organs were prepared and used for FACS analysis after staining with antibodies mentioned in Fig 17 and Fig.18.

The analysis of exosomes recovered in distinct hematopoietic / lymphoid organs after intravenous application strongly argued against unspecific capture and elimination of exosomes. Distinct from the in vitro uptake, exosomes were preferentially recovered in PEC, followed by BMC, SC and, less abundantly, PBL. They were hardly recovered in LNC. The uptake of exosomes differed in dependence on their origin. BMC preferentially took up AS-, PEC AS-Tspan8- and AS-Tspan8/CD9n- and SC AS-Tspan8- and AS-Tspan8-CD104-exosomes (Fig.17A). As demonstrated in Fig.17B and shown in detail in Fig17C, the target cell profile of leukocytes taking up in vivo the four types of exosomes differed. Fig.17C indicates the percentage of cells that express defined markers and have taken up exosomes. From there it became obvious that leukocytes expressing the tetraspanins CD63, CD81, CD151 and/or Tspan8 more readily take up AS-Tspan8 than AS exosomes. The same account for leukocytes expressing the integrin chains β1 (CD29), α6 (CD49f), β3 (CD61) and/or β4 (CD104) as well as for ICAM1 (CD54)$^+$, NCAM (CD56)$^+$, PECAM1 (CD31)$^+$, CD44s$^+$ and CD44v6$^+$ leukocytes. Exosomes are more readily taken up by myeloid cells (CD11b$^+$, CD11c$^+$) than by CD4$^+$ cells. They are rarely taken up by CD8$^+$ cells. Uptake of AS-Tspan8/CD9n exosomes by hematopoietic cells does not differ significantly from that of AS-exosomes. AS-Tspan8-CD104-exosomes have only an advantage in uptake by some of the leukocyte subpopulations that also preferentially take up AS-Tspan8-exosomes.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liver
pancreas
B
Lung
Liver
Pancreas
Large blood vessels
Kidney
Exosomes
AS
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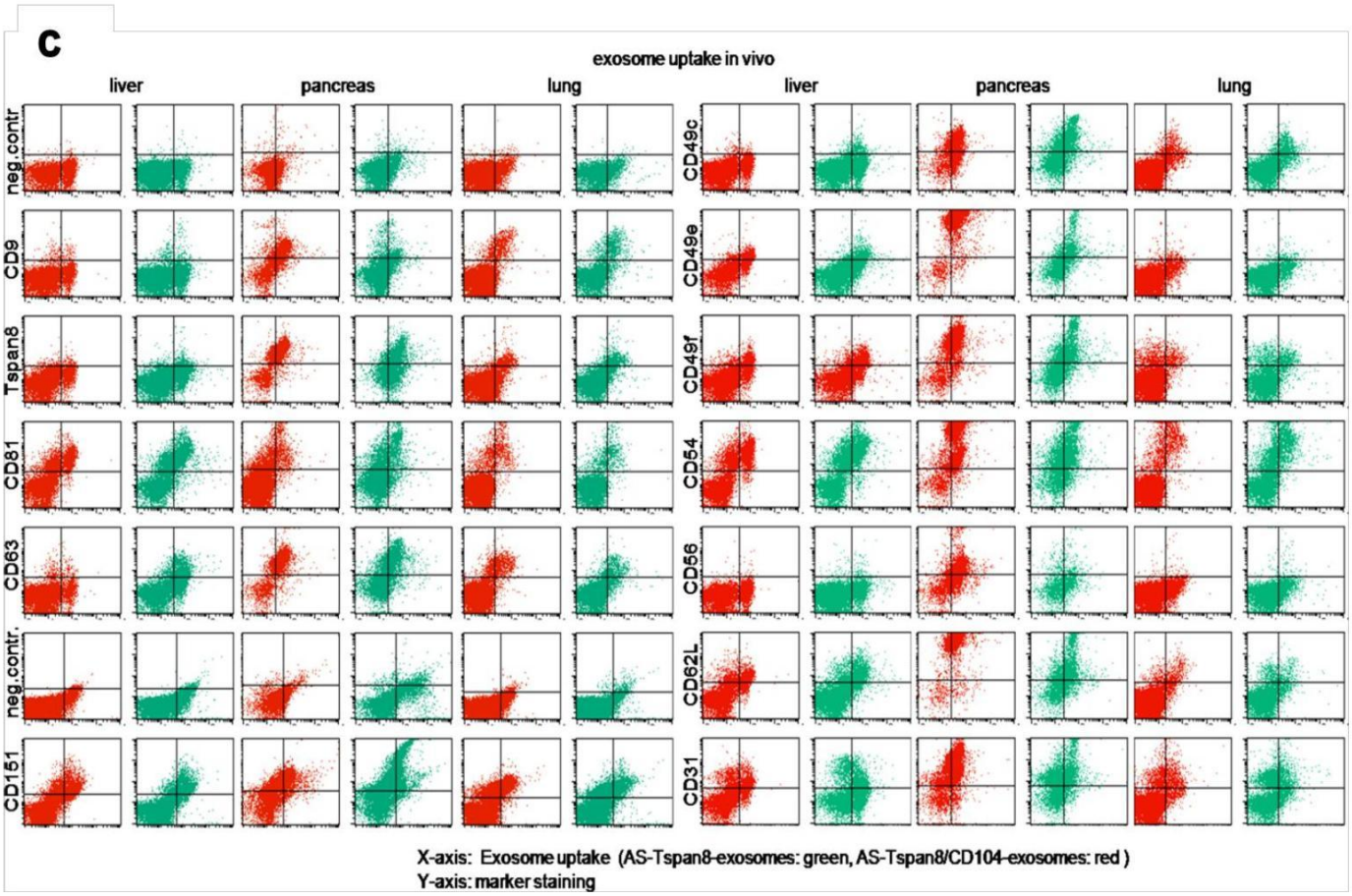
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CD63
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CD49e
CD49f
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CD62L
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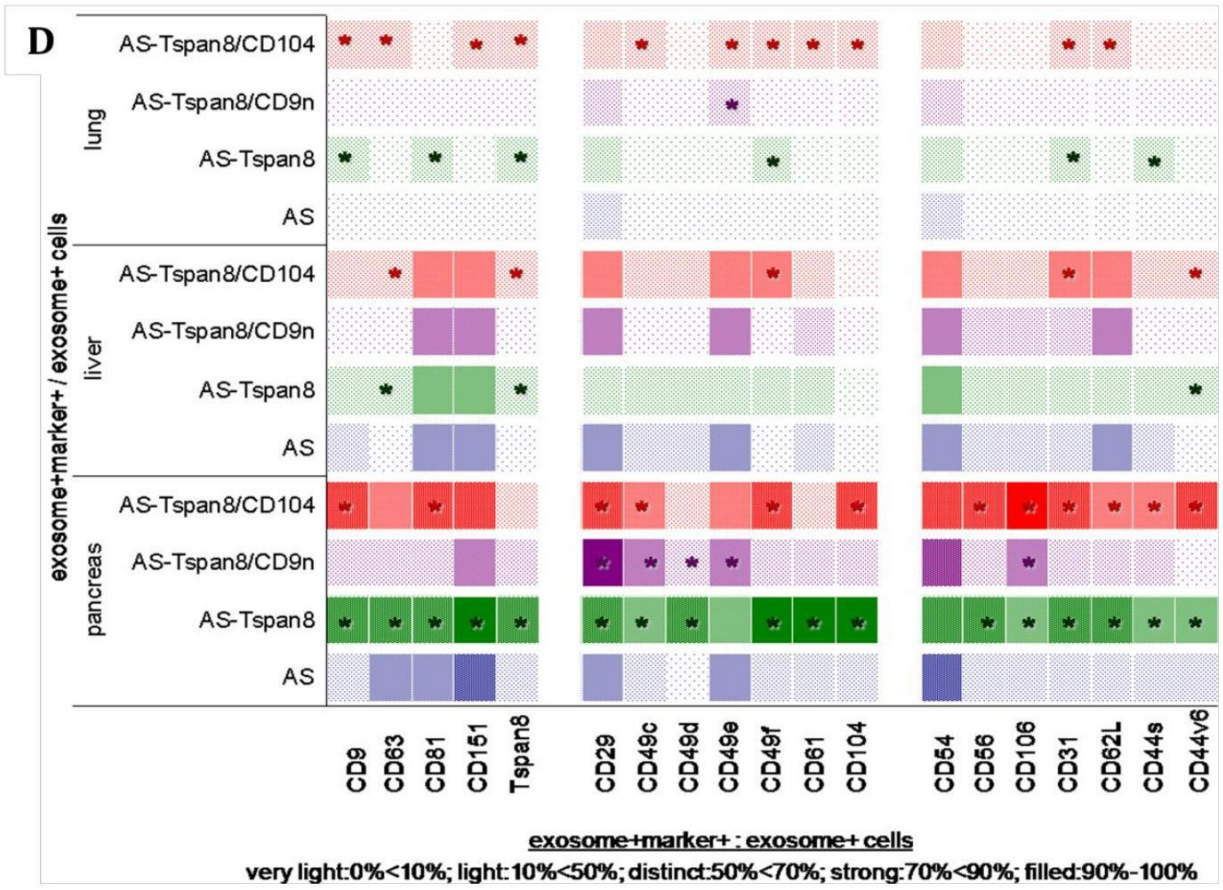
X-axis: Exosome uptake (AS-Tspan8-exosomes: green, AS-Tspan8/CD104-exosomes: red)
Y-axis: marker staining


D
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CD151
Tspan8
CD29
CD49c
CD49d
CD49e
CD49f
CD61
CD104
CD54
CD56
CD106
CD31
CD62L
CD44s
CD44v6
exosome+marker+ : exosome+ cells
very light:0%<10%; light:10%<50%; distinct:50%<70%; strong:70%<90%; filled:90%-100%

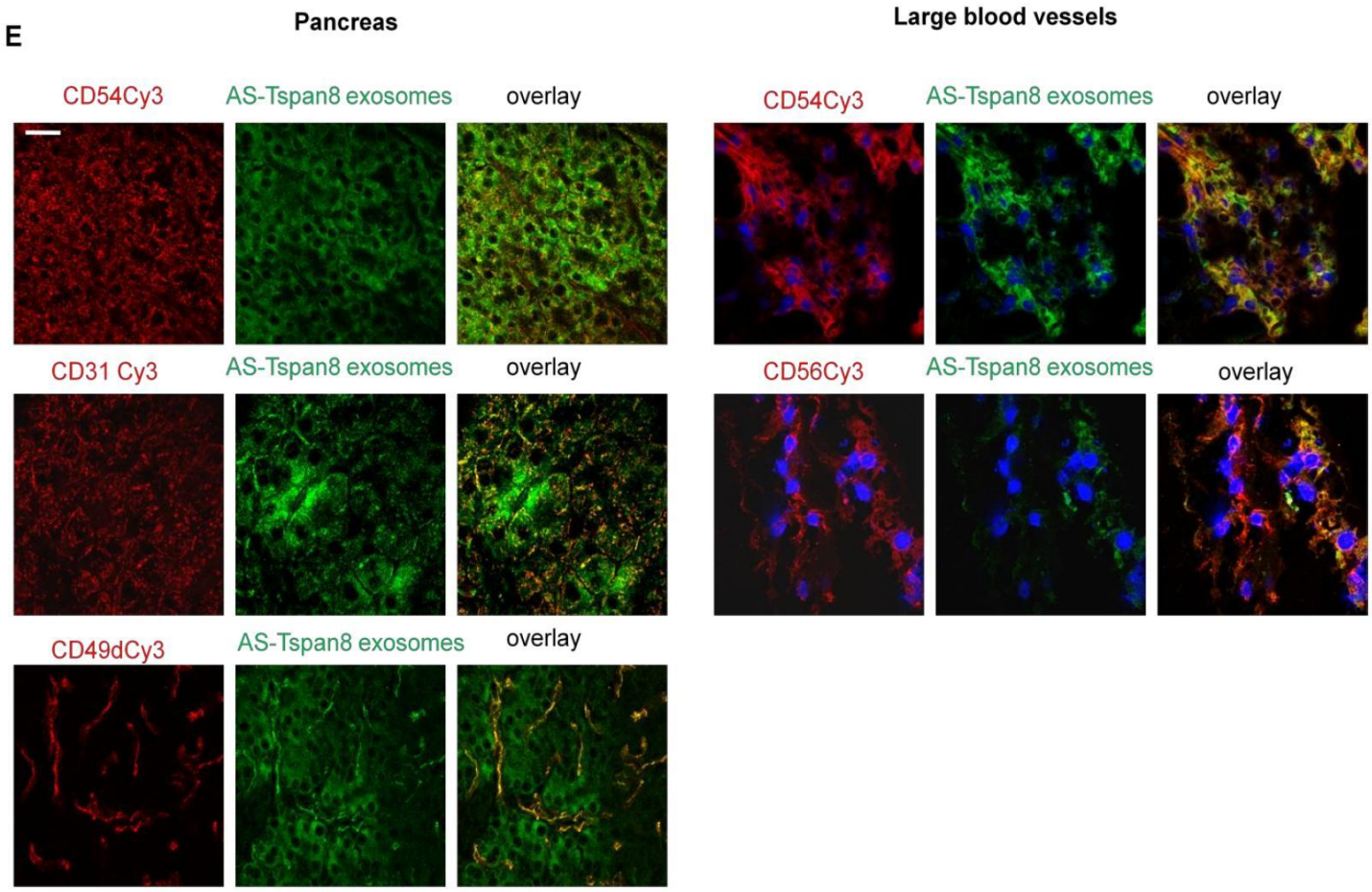

Figure 18. In vivo binding/ uptake of exosomes by solid organs. Solid organs were excised from rats as described in Fig.16 and were used for measuring the percentage of cells that took up exosomes, by flowcytometry of their single cell preparations (A), or shock frozen for histological studies (B). Sections were incubated with DAPI for nuclear staining (blue), and analysed by confocal microscopy to look for exosome uptake in vivo. The exosome signals are seen in green (B & E). To show the location within the organs, examples of transmitted light images (overlaid with green exosome signal) are provided below the dark field images (scale bar: 20µm). (C) Representative examples (flow cytometry). Cells in the upper left quadrant are marker$^+$, cells in the lower right quadrant are exosome$^+$ and the cell in upper right quadrant are marker$^+$ and have taken up exosomes. (D) Mean values of the percentage of double exosome$^+$, marker$^+$ cells/ exosome$^+$ cells in the indicated organs. The percentages of exosome$^+$ marker$^+$cells were grouped in negative (0-10%), weak co-staining (10<50%), distinct co-staining (50<70%), strong co-staining (70-90%) and (nearly) complete co-staining (90<100%). (E) Confocal microscopy of sections from pancreas and large blood vessels from rats receiving AS-Tspan8 exosomes. Sections were counterstained with the indicated antibodies and Cy3-conjugated secondary antibody. Marker staining (red), exosome uptake (green) and digital overlays are shown. Yellow indicates co-localization of exosomes with the indicated marker (scale bar: 20µm).

Surprisingly, 24h after intravenous application, exosomes were also recovered in solid organs, where striking differences were observed not only with respect to exosome uptake in general, but also with respect to the selectivity for distinct exosomes. Uptake/ binding by pancreas, liver and lung was evaluated by flow cytometry and immunohistology (Fig. 18). Transmitted light images were taken to get an idea of the localization withing the tissues. AS-Tspan8 exosomes were most readily taken up/ bound by the pancreas, likely in the exocrine region, followed by the spleen in possibly the B cell areas, large vessels (likely by endothelial cells, as also for AS-Tspan8/CD9n- exosomes) and the mesentery (in the yet un-

defined regions/cells). Few exosomes were recovered in the liver by possibly the kupffer cells, in the gut by, likely the crypt cells, and in kidney where they seem to be taken up by tubuli as shown with the help of transmitted light image overlaid with exosome signals in green (Fig18B). In the lung, exosomes seemed to bind to the tissue around the broncheoli and AS-, AS-Tspan8/CD9n-, and AS-Tspan8-CD104- exosomes were not taken up as readily. The most striking dfferences were seen in the large blood vessels, where different from AS-Tspan8- and AS-Tspan8/CD9n- exosomes, AS exosomes could only be found in what appears to be the peri-vascular tissue and, AS-Tspan8-CD104 exosomes were not detected at all. The latter were also not recovered in liver (Fig 18B). However, the precise regions/ cells which bind/ take up these exosomes in the tissues mentioned above have yet to be confirmed. In the first attempt to define the marker profile of cells taking up the distinct exosomes, isolated cells and tissue sections were counterstained with ICAM1, NCAM, PECAM and VCAM (CD106), as cells expressing these markers were found to have readily taken up exosomes according to flow cytometry analysis (Fig 18D). AS-Tspan8 exosomes were recovered in $ICAM1^{+}$cells in blood vessels and pancreas as well as $CD49d^{+}$ ($\alpha4^{+}$) endothelial cells (Representive examples are shown in Fig18E).

Taken together, it was shown that intravenously applied exosomes could find their target cells throughout the whole organism. We also demonstrated for the first time that exosomal tetraspanin web is decisive for exosome uptake by selective target cells *in vivo*. These findings could possibly be the first step towards creating tailored exosomes for therapeutic drug delivery to selected target cells.

4. Discussion

Exosomes are important contributors to the tumor cell communication with the environment (Schorey and Bhatnagar, 2008; van Niel et al., 2006). We and previous colleagues experienced that exosomes delivered by a Tspan8 cDNA-transfected tumor line, which constitutively expresses CD9 and CD151, promotes EC maturation and activation, which is initiated by exosomal Tspan8-associated CD49d binding to EC (Gesierich et al., 2006, Nazarenko et al., 2010). Based on this finding we asked, whether Tspan8, CD9 and CD151 recruitment into exosomes differs. Tspan8 internalization proceeds in a complex with INS2, clathrin and CD49d, but independent of CD9 and CD151. Besides the impact of AS-Tspan8-exosomes on EC, stimulation-induced internalization of a Tspan8-clathrin-INS2-CD49d complex is accompanied by loss in adhesiveness and pronounced motility.

Having resolved the internalization complex for Tspan8, I went further to explore if the changed tetraspanin associations during internalization are maintained on exosomes and if they have a role in exosomal target selection. I confirmed the selective binding of Tspan8-exosomes to endothelial cells and uncovered a strong prevalence for pancreatic cells. Importantly I show for the first time that exosomes applied *in vivo* are recovered in various organs throughout the body of the organism, and that the origin of the exosomes defines their targets.

4.1 Contribution of INS2 to Tspan8-internalization

Tspan8-internalization proceeds more rapidly than CD9- and slower than CD151-internalization. This is the consequence of a particular rearrangement of the Tspan8-web in response to stress, mimicked by PMA-treatment.

Tetraspanins can become internalized via an internalization motif, expressed by the tetraspanin or its associated proteins. Since the membrane proximity of the Tspan8 sorting motif, likely does not allow AP complex-binding (Berditchevski and Odintsova, 2007), we expected Tspan8 to co-internalize with, albeit weakly associated CD151. However, CD151 internalizes more rapidly than Tspan8 and the Tspan8-CD151 association becomes weaker on

PMA-treatment, which excludes Tspan8-internalization proceeding predominantly via CD151-association.

Tspan8 associates with EWI-F, INS2 and CD13 (Claas et al., 2005; in prep.). Weak co-immunoprecipitation of Tspan8 with CD13 becomes reduced during PMA-treatment excluding CD13 as the driver for internalization. EWI-F also associates with CD9 (Charrin et al., 2001; Claas et al., 2005) and remains CD9-associated during internalization, the association being far stronger than with Tspan8. Thus, it is unlikely that EWI-F contributes to Tspan8-internalization. In contrast, the weak Tspan8-INS2-association in resting cells is strengthened by PMA-treatment and Tspan8-, but not CD9- or CD151-internalization is severely impaired in AS-Tspan8-INS2^{kd} cells. INS2 interacts with the Tspan8 N-terminal region, Tspan8-internalization and co-immunoprecipitation with INS2 being strongly reduced after exchange of the N-terminal region including the first transmembrane pass. Intersectins contain SH3 and SH3A domains. SH3 domains interact with dynamin and synaptojanin (Yamabhai et al., 1998), where dynamin binding may regulate the fission process (Evergren et al., 2007, Koh et al., 2004). In fact, a dynamin inhibitor prevented Tspan8, but not CD9 internalization. SH3A domains are additionally involved in the intermediate step of coated-pit constriction (Simpson et al., 1999). The INS2 Eps15 homology domains promote endocytosis (Koh et al., 2007). Thus, the Tspan8-INS2 association can well account for pronounced Tspan8-internalization.

4.2 Tspan8-web rearrangement during PMA-treatment

In the resting state CD9 also, though weakly, associates with INS2. Therefore, activation-induced recruitment of additional molecules, which account for rearrangement of the Tspan8-web and pronounced internalization, became likely. In AS-Tspan8 cells, CD9, CD151 and Tspan8 are associated with CD49c. Only after PMA-treatment, Tspan8 co-localizes / co-immunoprecipitates with CD49d. In exosomes, Tspan8 is associated with CD49d, but not CD49c (Claas et al., 1998; Gesierich et al., 2005; Nazarenko et al., 2010). These findings suggested PMA-induced recruitment of CD49d in the Tspan8-web. Indeed, only after PMA-treatment Tspan8-clathrin-INS2-CD49d complexes are recovered in lighter density fractions, where they even resist partial cholesterol-depletion, known to dislodge tetraspanins into denser sucrose fractions (Zhang et al., 2009). Thus, during PMA-treatment Tspan8 associates

with CD49d and remains clathrin-associated, clathrin-coated pits being prone for internalization and known to support tetraspanin internalization (Helle and Dubuisson, 2008; Pols and Klumperman, 2009; Xu et al., 2009). As the CD49d association, which requires the large extracellular Tspan8 loop (Baldwin et al., 2008; Gutierrez-Lopez et al., 2003; Zhang et al., 2001), was weak in AS-Tspan8/CD9n-chimeras, it becomes likely that Tspan8-internalization requires both INS2 and CD49d, where CD49d possesses an internalization motif (Pandey, 2009).

Internalized membrane microdomains are recruited into early endosomes from where they can proceed along different pathways (Cocucci et al., 2009; deGassart et al., 2004; Février and Raposo, 2004; Johnstone, 2006; Stahl and Barbieri, 2002). These pathways clearly differ between Tspan8 and CD9. Tspan8, distinct from CD9, is mainly recovered in early endosomes. It is also recovered in $LAMP1^+$ vesicles, which can derive from late endosomes or the synthetic pathway (Chapuy et al., 2008; Lakkaraju and Rodriguez-Boulan, 2008). Because the Tspan8-CD49d complex is abundantly recovered in exosomes, although Tspan8 does not associate with CD49d during biosynthesis (Claas et al., 2005), Tspan8 in $Lamp1^+$ vesicles likely belongs to the recycling pool. How Tspan8 becomes recruited in $Lamp1^+$ vesicles remains to be clarified. Finally, we suggest that the Tspan8-CD49d-association accounts for the different fate of internalized Tspan8 and CD9. Jones et al. (2006) described different routes of integrin recycling via a short and a long loop, where short loop recycling requires PKD1 downstream of Rab4. CD49d is shown to undergo rapid recycling (Rose, 2006; Potapova et al., 2008). Our data points to CD49d-associated Tspan8 taking the short loop for release.

Thus, activation-induced internalization (and exosome release) of Tspan8 depends on the association with INS2 and CD49d. As Tspan8-internalization is significantly retarded after INS2 silencing or exchange of the N terminal region, INS2 likely is involved in the fission process. A PMA-induced membrane microdomain re-organization promotes proximity between Tspan8 and CD49d, such that the CD49d internalization motif can facilitate recruitment into endosomes.

4.3 Pronounced Tspan8-CD49d-internalization impairs adhesion and promotes motility

The particular route of PMA-induced Tspan8 internalization has functional consequences. As the internalization motif of the CD151 C-terminal tail could skew functional activities of AS-Tspan8/CD151c-chimeras, only data on AS-Tspan8 and AS-Tspan8/CD9n are discussed.

Tspan8-promoted FN- and Ln5-adhesion was abolished in AS-Tspan8/CD9n and strongly reduced in PMA-treated AS-Tspan8 cells. In line with these findings, D6.1 and anti-CD49d, but not anti-CD49c, became inefficient in blocking adhesion of PMA-treated AS-Tspan8 cells. AS-Tspan8 cells / exosomes efficiently adhere to EC, binding being inhibited by D6.1 and anti-CD49d, but not anti-CD49c (Nazarenko et al., 2010). In line with pronounced PMA-initiated internalization of CD49d-associated Tspan8, PMA-treated AS-Tspan8 cells poorly adhered to EC and adhesion was inefficiently inhibited by D6.1 or anti-CD49d.

Pronounced PMA-induced Tspan8-CD49d internalization is accompanied by increased motility, which depends on the intact Tspan8 N-terminal region. Anti-CD49d and D6.1, but not anti-CD49c inhibiting AS-Tspan8 migration strengthened the importance of the Tspan8-CD49d-association.

Internalization, recycling and/or release of exosomes are rapid processes such that, demonstrated for in vitro wound healing, PMA-initiated effects could vanish during a prolonged assay period. Therefore, cable formation was evaluated in the absence of PMA. Though the capacity for cable formation is maintained in AS-Tspan8/CD151- and -/CD9c-chimeras, the majority of cells grow in clusters. AS-Tspan8/CD9n- and -/CD9c+n-chimeras largely lost cable formation capacity, which confirms the importance of the Tspan8 N-terminal region for exhibiting morphogenic features. The underlying mechanism remains to be explored.

AS-Tspan8-exosomes strongly promote EC maturation and activation (Nazarenko et al., 2010) and, also advantageous for metastasizing tumor cells, Tspan8 internalization strengthens motility and cable formation. We here demonstrate that these Tspan8-selective activities rely on an activation-induced association of Tspan8 with INS2 and CD49d accompanied by a rearrangement of the Tspan8-web, where Tspan8 becomes recruited into

internalization-prone membrane microdomains. Elucidating the path of exosome generation clearly is one of the first steps in understanding exosome-target cell interaction.

Exosomes are suggested to provide a most powerful therapeutic tool, owing to their capability of transferring functionally active proteins, mRNA and miRNA, which allows for striking target cell modulation (Denzer et al., 2000, Février and Raposo, 2004; Valadi et al., 2007, Iero et al., 2008). Accordingly, exosomes are suggested to provide a most powerful therapeutic tool (Viaud et al., 2010; Seow et al., 2009), which was confirmed in our lab. A rat tumor model where exosomes express Tspan8, sufficed for overshooting angiogenesis induction such that rats became moribund due to disseminated intravascular coagulation despite that the exosome delivering tumor was located subcutaneously (Claas et al., 1998). This demonstrates not only the power of exosomes, but also the danger posed by inappropriate exosome delivery. Appreciating the potential therapeutic as well as life threatening power of these particular vesicles (Porto-Carreiro, 2005; Vella et al., 2008) which are found in all body fluids (Mathivanan et al., 2010), it became demanding to explore, (i) whether exosomes use defined structures to bind to and fuse with target cells and (ii) whether exosomes require special ligands to recognize a potential target cell. In the second part of my thesis I demonstrated that tetraspanins and tetraspanin-associated molecules are decisive for exosome binding and uptake. In addition, exosomes diffuse through the whole body, but are very selectively enriched in distinct cells / organs depending on their origin / tetraspanin-complex and apparently through the appropriate ligand expression of target cells / tissues. This exquisite selectivity of exosomes should, indeed, allow for their therapeutic use.

4.4 The exosomal tetraspanin web

The finding that the Tspan8 web is changing during internalization such that the association with CD9 and CD151 becomes weakened and that with CD49d strengthened, led us to suggest that individual cells may deliver a set of distinct exosomes. However, when exosomes became bound to latex beads via D6.1, the majority of exosomes bound and the remaining exosomes were equally well depleted for Tspan8$^+$ and CD9$^+$ exosomes. However, anti-CD9-coated beads retained only part of the exosomes and more efficiently CD9$^+$ than Tspan8$^+$ exosomes. From there we concluded that possibly due to the ongoing process of exosome generation by internalization as well as by vesicle formation during synthesis (Simons and

Raposo, 2009; Blanc et al., 2010) at least the majority of exosomes cannot be differentiated based on distinct tetraspanin expression. The large size difference between latex beads (4μm) and exosomes (0.03- 0.1μm), may provide an additional hindrance in the isolation of subtypes of exosomes via antibody-coated latex beads. Nonetheless, the subtle differences observed with anti-CD9-coated beads support our hypothesis that individual cells can deliver distinct exosomes.

Exosomes are suggested to bind selective targets (Lakkaraju et al., 2008; Johnstone, 2006) and our group has shown before that this depends on exosomal tetraspanins (Gesierich et al., 2006; Nazarenko, 2010). As we could not reliably separate exosome subtypes, it became likely that exosomes select for their target cell via tetraspanin-complexes, rather than via individual tetraspanins. This is well in line with tetraspanins acting as molecular facilitators that mostly modulate activity of associated molecules (Levy et al., 2005; Hemler, 2005).

4.5 Exosome selectivity for target cells

Exosomes express phosphatidylserine (PS) on their surface (Thery et al., 2002) which may provide an "eat me" signal (Schlegel et al., 2001) and has been suggested to act as the exosome binding receptor (Smalheiser, 2007; Simons and Raposo, 2009; Simpson et al., 2009). However, it also has been shown that AnnexinV does not suffice to block exosome uptake by natural killer (NK) cells indicating that presence of phosphatidyl-serine alone is not sufficient for target cell binding (Keller et al., 2009) and requires additional signal. In fact, particularly the strong *in vitro* binding of exosomes to monocytes ($CD11b^+$) in the peritoneal exudate suggests a contribution of scavenger receptors to exosome binding via phosphatidyl serine. However, the selectivity of exosome binding argues against a major role of PS in exosome uptake and, indeed, exosome uptake in vivo was not dictated by scavenger receptors. Thus, PS likely facilitates unspecific binding that may not be followed by exosome uptake.

Exosomes derived from AS. AS-Tspan8, AS-Tspan8/CD9n and AS-Tspan8-CD104 cells, which differ only in Tspan8 and CD104 expression were tested in vitro for their selectivity of target cell binding. Clearly, these minor differences sufficed for distinct binding to hematopoietic cells, non-transformed and tumor cells. We confirmed the preferential binding of AS-Tspan8 exosomes to endothelial cells. Concerning hematopoietic cells, AS-Tspan8

exosomes preferentially bound to BMC and LNC. In contrast, AS-derived exosomes have a selective advantage for LuFb; AS-Tspan8-CD104 exosomes preferentially bind LnStr, tumor cells and LNC. As demonstrated by the in vitro binding of AS-Tspan8 exosomes, these exosomes preferentially bind target cells expressing CD11b/CD18 (hematopoietic cells) or CD54, CD44, CD31 or CD62L (hematopoietic and endothelial cells).

The more fascinating finding was the target cell selectivity of exosomes in vivo, where binding / uptake differed significantly for the 4 exosome preparations. First to note, the recovery of exosomes in vivo did not in all instances overlap with in vitro binding features, indicating that on the journey through the body, they become selectively trapped. In line with this, after i.v. injection, exosomes are not trapped in the first capillary bed. They are also not recovered preferentially in organs destined for degradation or release like spleen, liver and later on the kidneys. Instead, exosomes from the 4 lines have different preferences. AS-Tspan8 exosomes are preferentially found in the pancreatic gland, the spleen and large vessels. Though AS-exosomes are also recovered in large vessels, it could be said from the morphology that they seemed to associate with different cells.

In other organs, like the liver and the gut, few exosomes from all 4 lines are recovered. AS and, most pronounced AS-Tspan8-CD104 exosomes are also bound to broncheolar epithelial cells. Counterstaining with selective tissue markers so far unraveled that $CD54^+$ and $CD31^+$ epithelial and mesenchymal cells preferentially take up exosomes. Co-localization as revealed by confocal microscopy pointed towards some of the molecules seen on cells taking up exosomes to be potentially the actual ligand(s).

To address the question of ligand involved in exosome binding, studies via pull down assays are underway, where either cells or exosome lysates serve as matrix-bound catcher. This will allow the final proof of the engaged tetraspanin-complex receptors and their target cell ligands. Whether the exosomal tetraspanin web accounts for focalizing/ activating the receptor or whether tetraspanin, in addition, are involved in the internalization process remains to be explored. The latter could well be the case since, it has been suggested that the internalization process in the exosome donor cell and exosome uptake by target cell may follow the same fission/ fusion machinery (Ahmed and Xiang, 2010). If this is the case, the particular route of Tspan8 internalization may add to the selectivity of target cells. Still, the unexpected strong

organ selectivity in vivo requires additional experiments, as it becomes likely that chemokines / chemokine receptors within the exosomes and the targeted tissue come into play.

5. Conclusion and Outlook

Exosomes are most important intercellular communicators. It is known that exosomes abundantly express tetraspanins as one of their constitutive components. Tetraspanins, on the other hand, are known as molecular facilitators that modulate the activity of associated molecules and are involved in fusion and fission processes (Hemler, 2008). I here provided for the first time convincing evidence for exosomal activity of tetraspanins.

First, tetraspanins, as demonstrated for Tspan8, are engaged in exosome composition via their route of internalization and the rearrrangment of their web of associating molecules. Notably, different tetraspanins use distinct paths of internalization. Furthermore, the available potential partnering molecules exert a considerable impact on exosome assembly and thereby on target cell selection. Extending the exploration of the internalization path to other tetraspanins and their associating molecules may greatly improve therapeutic efficacy of exosomes, e.g. in immunotherapy of cancer, where dendritic cell-derived exosomes are already in use as a potent means to induce a tumor antigen-specific T cell response (Viaud et al., 2010).

Second, tetraspanins contribute to exosomal target cell selection. Selective targeting of exosomes has been suggested repeatedly (Belting and Wittrup, 2008; Mallegol et al., 2007; Schorey and Bhatnagar, 2008; Simons and Raposo, 2009), but has not yet been proven. Previous work in our group pointed towards tetraspanins (Tspan8) to be involved in the target cell selection process. I demonstrated that this is indeed the case, where the selectivity of exosome binding / uptake could be demonstrated most convincingly in vivo, where exosomes apparently reach their target cell throughout the body, irrespective of where they are generated. Again, the tetraspanin complexes appear to be the decisive element. The work for defining the ligands on various tissues and their partners in the tetraspanin complexes is underway and is being addressed by coupling lysates of exosomes to activated-sepharose matrix, which would ‘catch’ the ligands from lysates of cells of interest when passed over the coupled matrix. In a reverse setting, molecules on exosomes that act like receptors for the target cell ligands can be identified.

This knowledge will be helpful in generating tailored exosomes by equipping them with tetraspanin and associating receptors that allow to selectively target any cell type of interest

for therapeutic agent delivery. We are currently exploring this possibility in a model system of "tailored" exosomes delivered by non-transformed lung fibroblasts. Application of such exosomes compared to those from untransfected fibroblasts will provide answers to whether such tailored exosomes can specifically deliver any therapeutic message which could be cytotoxic drug, oncogene-suppressing miRNA, tumor growth suppressive agent, they carry to target cells. If our hypothesis holds true, it will prove to be a boon to therapeutics since, such an approach will have advantages of minimum cross- interaction with and harm to other cells. Use of tailored exosomes with defined specificity will not just be limited to field of cancer, but can also be applied for gene therapy of some genetic disorders like diabetes and cystic fibrosis and autoimmune diseases since they would be an ideal vehicle which will be non-toxic, stable *in vivo* and easily engineered.

The observation that exosomes can transfer proteins, mRNA and miRNA to other cells that are functional in the new environment is exciting and heralds an unanticipated and until recently considered impossible mode of cell-cell communication, which creates hope for new most potent therapeutic options. I am confident that building on our previous work further unraveling the contribution of exosomal tetraspanins in physiological and pathological processes will provide valuable hints towards exosome-based therapeutics.

6. Summary

Exosomes are deemed to be the most important intercellular communicators and tetraspanins / tetraspanin-complexes are suggested to play an important role in exosomal target cell selection. It has been shown that only exosomes expressing a Tspan8-CD49d complex preferentially bind endothelial cells, which initiates angiogenesis. This finding was unexpected as in the exosome producing cells, Tspan8 is associated with CD49c and the tetraspanins CD9 and CD151. Considering the discussed therapeutic power of exosomes as message / drug transporter, it became important to clarify the mechanisms accounting for the distinct Tspan8 web in the cell membrane versus exosomes. We therefore compared the route of Tspan8 and Tspan8-chimera internalization, where the N- and/or C-terminal regions were exchanged with the corresponding regions of CD9 or CD151.

Activation-induced Tspan8 internalization indeed proceeds more rapidly than CD9 internalization and is accompanied by disassembly of the Tspan8-CD9-CD151 membrane complex found in the resting cells. Tspan8-internalization relies on the association of the Tspan8-N-terminal region with intersectin-2, part of a multimodular complex involved in clathrin-coated pit internalization. Internalization and recovery of Tspan8 in early endosomes is further promoted by the recruitment of CD49d such that only in PMA-activated cells a Tspan8-INS2-CD49d-clathrin complex is recovered in cholesterol depletion-resistant membrane microdomains. PMA-induced Tspan8-internalization promotes cell migration, but reduces matrix and cell adhesion.

Suggesting that this particular route of Tspan8 internalization contributes to exosomal Tspan8 web as well as selective activities of Tspan8-expressing exosomes, I proceeded with

a comparative analysis of the exosomal tetraspanin web aiming for definition of exosomal target cells.

Co-immunoprecipitation of cell lysates and exosomes from 4 rat tumor lines, AS, AS-Tspan8, AS-Tspan8/CD9n and AS-Tspan8/CD104, which express CD9, CD81, CD151 and the CD49c and CD49d integrin chains, but differ by Tspan8 and CD104 integrin chain expression revealed that CD9, CD81 and CD151 preferentially associate with CD49c and, only CD151 with CD104 in cell lysates as well as exosomes. Instead, Tspan8 associated with CD49c in cell lysates, but with CD49d and CD104 in exosomes. The differences in complexes on exosomes severely influenced target cell selection in vitro and in vivo, where the latter revealed efficient exosome uptake in different hematopoietic compartments and solid organs after i.v. injection. Only exosomes expressing the Tspan8-CD49d complex preferentially integrated into endothelial cells and pancreatic tissue, exosomes expressing the CD151-CD104 or the Tspan8-CD104 complex were preferentially taken up by stromal cells in liver and lung, exosomes expressing CD9- or CD81-CD49c complexes were mostly recovered in hematopoietic cells.

Taken together, we clarified the route of Tetraspanin8 internalization and showed that the changed associations created during internalization are maintained in exosomes. Moreover, the differences in tetraspanin complexes, which are evident from the point of internalization as well as in the exosomes, allow for the selectivity of exosomes towards target cells. These findings provide for the first time convincing evidence that exosomal tetraspanins contribute to target cell selection such that, according to the tetraspanin-complex on exosomes predictions can possibly be made on potential target cells. This knowledge will allow generating tailored exosome for drug delivery.

7. References

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Publications

1. **Rana S**, Dringen R. 2007. Gap junction hemichannel-mediated release of glutathione from cultured rat astrocytes. *Neurosci Lett*

2. **Rana S**, Claas C, Kretz CC, Zöller M. 2010. Activation-induced internalization differs for the tetraspanins CD9 and Tspan8: Impact on tumor cell motility. *Int J Biochem Cell Biol.*

3. **Rana S**, Zöller M. 2010. Exosome target cell selection and the importance of exosomal tetraspanins: a hypothesis. *Biochem Soc Transaction* (in press).

4. Nazarenko I, **Rana S**, Baumann A, McAlear J, Hellwig A, Trendelenburg M, Lochnit G, Preissner KT, Zöller M. 2010. Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation.

Abstracts

1. **Sanyukta Rana**, Irina Nazarenko, Margot Zöller (2009). Differences in the impacts of D6.1A, CD9 and CD151 tetraspanins on cell motility and adhesion rely on distict modes of their internalization. Graduate seminar, PhD retreat (Weil der Stadt, 16- 21 July 2009).

2. **Sanyukta Rana**, Irina Nazarenko, Margot Zöller. The contribution of the N- and C-terminal tails of Tspan8 to TEM organization and internalization. 3rd European Conference on Tetraspanins, “Tetraspanins as potential therapeutic targets” (Villejuif, Paris, 10-11 September 2009).

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Educational Background

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Masters in Biochemistry and Molecular Biology

- Studying at University of Bremen, Germany
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- **Major modules** in masters: Basic aspects of Biochemistry, Molecular biology, Cell biology and Microbiology, Bio-organic chemistry.
- **Supplementary modules:** Basic module in Biochemistry and Molecular biology, Basic module in Medicinal chemistry, Basic module in Signal transduction, Metabolism in brain cells.
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Lab-rotations:

Each lab rotation was for 6 weeks period, in different departments, to learn various techniques and ways to handle different kinds of scientific challanges.

March 2005	Studying the interaction of BSH and DMPC liposomes for a more effective BNTC (Boron Neutron Capture Therapy) for cancer.
May 2005	Phylogenetic analysis of clam species- *Tridacna maxima,* for planning an efficient conservation program for this endangered species.
August 2005	Transposon mutagenesis bank and quorum sensing assays, to study Quorum sensing in bacterium Azoarcus.

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Laboratory Work Experience
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2002 Centre for Biochemical Technology, Delhi	• Trained for a month with a team of scientists working on Diabetes (type 2).
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German	Conversational	Basic	Basic
Hindi (mother tongue)	Fluent	Very Good	Very Good
Sanskrit	Good	Very Good	Very Good