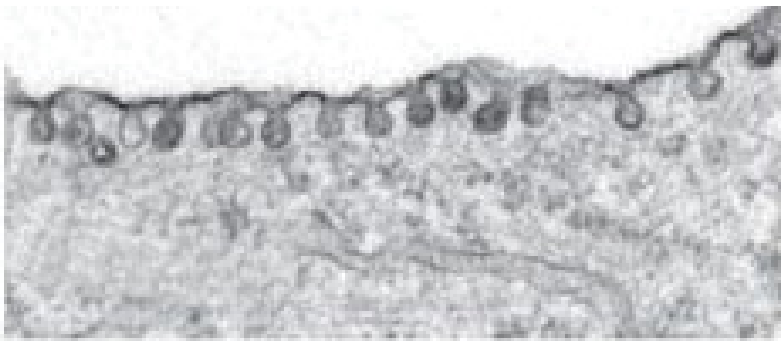
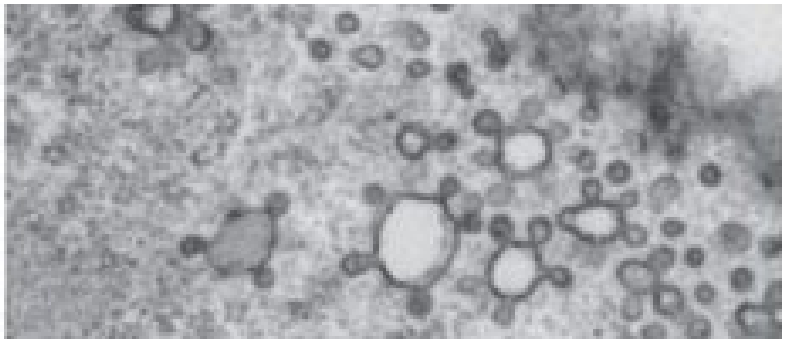
# Caveolae and cancer

## The controversial role of caveolin-1





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**About the cover:**

The figure on the cover shows electron micrographs of caveolae in adipocytes. Caveolae are visualized by labeling the surface using an electron-dense marker. Caveolae are found in different forms, like single caveolae (left panel) and grapelike structures (right panel). Adapted from (Parton and Simons, 2007)

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**Abstract**

More than fifty years ago caveolae were discovered and marked as a new endocytic mechanism. Later it was found that they are formed in microdomains of the plasma membrane, called lipid rafts. The caveolar endocytotic route is known to transport several molecules like, membrane components and growth receptors into the cell. The main functional components of caveolae are the caveolins, caveolin-1, caveolin-2 and caveolin-3. These proteins are not only necessary for caveolae formation and dynamics, but are also involved in the regulation of several signaling molecules. Caveolae may play a role in tumorigenesis. In this thesis we aim to discuss the recent progress in our understanding of the role of caveolins in cancer. Caveolin-1 is known to act as both, a tumor suppressor and an onco-protein, here we will explain how this difference is established.

**List of Abbreviations**

| | |
|---|---|
| CD | Cluster of Differentiation |
| CLIC-D | Dynamin-dependent clathrin-independent carriers |
| CLIC-DI | Dynamin- and clathrin-independent carriers |
| CSD | Caveolin scaffolding domain |
| eNOS | endothelial nitric oxide synthase |
| EGF | Epidermal growth factor |
| ER | Endoplasmatic reticulum |
| ER-$\alpha$ | Estrogen receptor |
| FAK | Focal adhesion kinase |
| GPCR | G-protein-coupled receptors |
| GPI | Glycosylphosphatidylinositol |
| GTP | Guanosine triphosphate |
| IAP | Inhibitor of apoptosis proteins |
| IGF-1 | Insulin like growth factor |
| IL | Interleukin |
| IRS1 | Insulin receptor substrate 1 |
| LEF | lymphoid enhancer factor |
| LGMD-1C | Limb-girdle muscular dystrophy |
| LOH | Loss of heterozygosity |
| MDCK | Madin Darby canine cells |
| MEDF | Mouse embryonic dermal fibroblasts |
| MEF | Mouse embryonic fibroblasts |
| MHC | Major histocompatibility complex |
| MURC | muscle-restricted coiled-coil protein |
| OHT | 4-hydroxytamoxifen |
| PDGF | Platelet derived growth factor |
| PI3-kinase | Phosphatidylinositol 3-kinase |
| PTRF | Polymerase I and transcript release factor |
| RT-PCR | Real time – polymerase chain reaction |
| SDPR | serum deprivation response |
| SH2 | Src homology domain |
| SRBC | SDR-related gene product that binds to C kinase |
| SV40 | Simian virus 40 |
| TRAMP | Transgenic adenocarcinoma of mouse prostate |
| TCF | T-cell factor |
| VIP21 | Vesicular Integral-membrane Protein of 21 kDa |

| | |
|---|---|
| A | Alanine |
| F | Phenylalanine |
| H | Histidine |
| I | Isoleucine |
| L | Leucine |
| R | Arginine |
| T | Threonine |
| Y | Tyrosine |
| W | Tryptophaan |

**Index**

## 1. Introduction

To internalize molecules from outside the cell, cells use a process called endocytosis. Via this process, cells can take up macromolecules, proteins and ligands. Besides extracellular particles, cellular molecules such as transmembrane receptors, membrane proteins and lipids can be internalized (Miaczynska and Stenmark, 2008). By taking up of these molecules, a cell can control the composition of the plasma membrane and cellular processes, like nutrient uptake, signal transduction, cell migration and cell adhesion (reviewed in (Grant and Donaldson, 2009).

There are several endocytic routes known (figure 1). The best described route is the clathrin-mediated endocytic pathway. A hallmark of this pathway is the coat composed by clathrin and adaptor proteins. It is formed around the invagination, and which can be observed by electron microscopy (Wilson et al., 1989). A variety of clathrin-independent routes is involved in the uptake of particles and membrane components into the cell. Examples are phagocytosis, caveolae-mediated endocytosis, macropinocytosis, dynamin-dependent clathrin-independent carriers (CLIC-D) and dynamin- and clathrin-independent carriers (CLIC-DI) (figure 1) (Mosesson et al., 2008). Phagocytosis is involved in the uptake of large particles, like vesicles of dead cells or pathogens, and is mostly used as a defense mechanism by specialized cells, such as macrophages (Huynh et al., 2007). The other pathways are involved in the uptake of smaller cargo. This mostly occurs via invaginations of the plasma membrane, after which the vesicle is formed after fission (with or without the involvement of dynamin). However, macropinocytosis does not act via an invagination, but via membrane ruffles (figure 1) (Swanson and Watts, 1995).

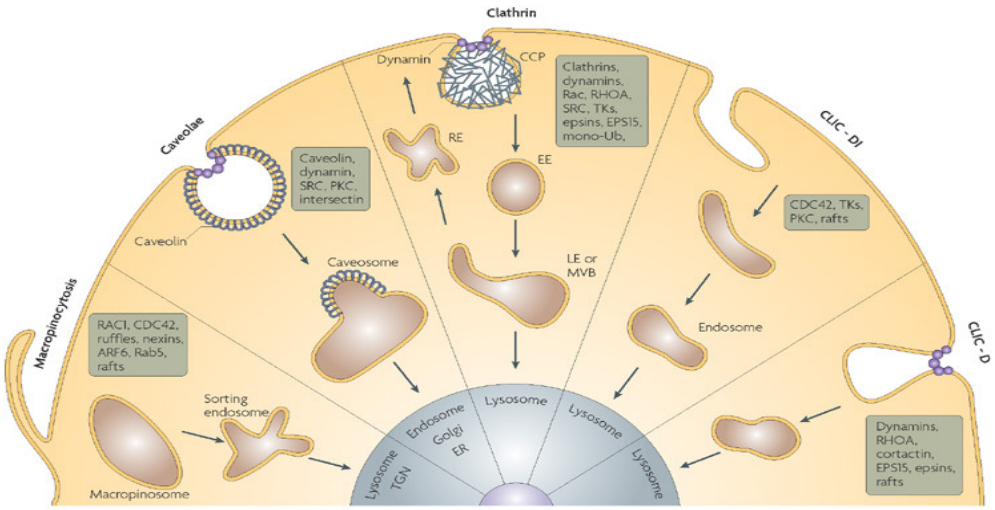


Figure 1: Schematic overview of endocytic routes. The five major endocytosis routes are depicted in the diagram. The different modes of transportation of vesicles and cargo are illustrated and which proteins are known to be involved (Mosesson et al., 2008).

Clathrin-mediated endocytosis occurs in mammalian cells and can be used to internalize nutrients, growth factors and receptors. For example, iron can be internalized via clathrin-coated vesicles, when iron-bound transferrin binds to the transferrin receptor that is endocytosed via the clathrin-mediated route. Cargo selection in clathrin-coated pits is mediated via specific adaptor proteins like TPP and β-arrestin. These adaptor proteins will target the cargo to clathrin-coated pits (Traub, 2005). Invagination of a clathrin-coated vesicle is mediated by clathrin triskelions, which connect with each other to form a basket (Greene et al., 2000). When a pit is formed, the GTPase dynamin will be recruited to the neck of the invagination. Dynamin will form a helical polymer around this neck. Upon GTP hydrolysis, dynamin will mediate fission of the vesicle from the plasma membrane (Oh et al., 1998).

Upon clathrin-mediated endocytosis, the vesicles fuse with an early endosome (figure 1). The early endosome is often located near the plasma membrane. Through its mildly acid pH, an early endosome can function like a sorting organelle (figure 2). Because of the low pH, ligands can be dissociated from receptors (Mellman, 1996). However, signaling from activated receptors can sometimes continue even when the ligand and receptor are separated, therefore degradation of the receptors in the lysosome is sometimes required to terminate signaling (Baass et al., 1995). After the sorting of ligands, nutrients and receptors in the endosome, proteins can either go to an endosomal recycling compartment to be transported back to the plasma membrane or traffic to the late endosome and subsequently to the lysosome (figure 2) (reviewed in (Maxfield and McGraw, 2004). The lysosome contains hydrolytic enzymes, like proteases, nucleases and lipases required for degradation of proteins and lipids. These enzymes are transported from the Golgi complex to the lysosome in a pro-enzyme conformation, meaning they have low enzyme activity and are modified with sugar moieties. The lysosomal enzymes are active in a milieu with a pH of 5. This mechanism protects cellular proteins if these enzymes escape from the lysosome. Once the enzymes are activated, they can cleave substrates, whereby the substrates are degradated (Kornfeld, 1990).

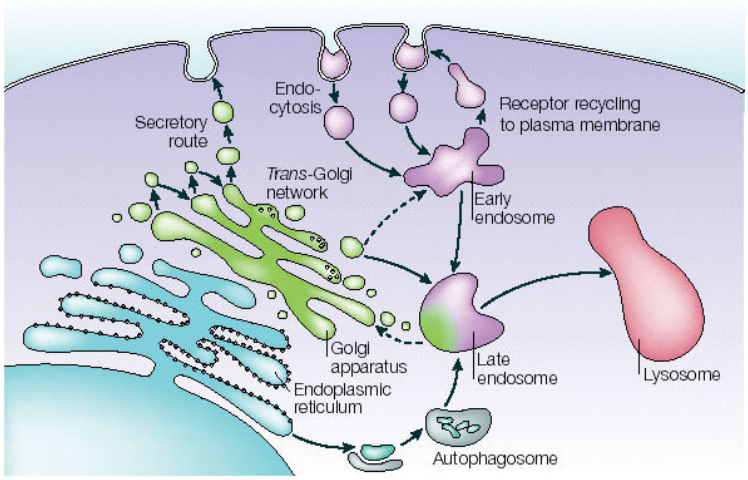


Figure 2: Schematic overview of trafficking in a cell. The classical endocytic route will lead via the early endosome to the late endosome and finally to the lysosome where degradation will take place. The early endosome functions as a sorting organelle, molecules can go to the late endosome, or recycle to the plasma membrane. Adapted from (Jeyakumar et al., 2005).

Besides the clathrin-dependent endocytic pathway, caveolae-mediated endocytosis is gaining more and more understanding. It is now known that caveolin-1 is one of the functional components of caveolae (Rothberg et al., 1992), and later also other proteins are discovered, like caveolin-2 (Scherer et al., 1996), caveolin-3 (Tang et al., 1996) and cavin-1 (Jansa et al., 1998). Contrary to the classic endocytic route, trafficking of endocytic caveolae will not lead to the early endosome, but to the caveosome. The function of this compartment is similar to the early endosome, since it can sort proteins, however its pH is neutral (Kirkham et al., 2005). The trafficking route of caveolae will be discussed in chapter 4.

This thesis will review our current understanding of the role of caveolae and caveolin proteins in cancer. In The Netherlands, cancer became the number one cause of death in 2008, with 30% of all causes of death being due to cancer (CBS). Since twenty years it has been suggested that caveolae and caveolin are involved in cancer. This thesis will focus on the involvement of caveolin-1 in cancer. The trafficking and function of caveolae will be highlighted. Caveolin is one of the major components of caveolae, and therefore the structure of this protein will be discussed. The idea that caveolin would be involved in cancer came from a study investigating which proteins were being phosphorylated in Rous sarcoma virus transformed cells (Glenney and Zokas, 1989). In chapter 6 an overview of the different roles of caveolin-1 in cancer will be given.

Cancer cells have other characteristics than normal cells. They are able to replicate limitlessly, are self-sufficient in their growth signals, evade apoptosis and can migrate to other parts of the body to form metastases (Hanahan and Weinberg, 2000). Caveolin may be involved in many of these

characteristics, in chapter 7 is discussed how caveolin-1 may drive tumorigenesis via these the different processes.

## 2. Caveolae

### Identification of caveolae

More than 50 years ago caveolae were identified by G. Palade (Palade, 1953). Using electron microscopy he described invaginations (pits) at the plasma membrane of heart endothelial cells. They were named: *caveolae intracellulares* (Yamada, 1955), which means 'little caves' in Latin. Caveolae are approximately 70 nanometer (nm) in diameter and have an omega-shaped structure (figure 3) (Palade and Bruns, 1968). Caveolae do not have an electron-dense coat, like clathrin-coated pits have, as shown in figure 3 (Stan, 2005). They are implicated in endocytosis, transcytosis and signal transduction (Lisanti et al., 1995).

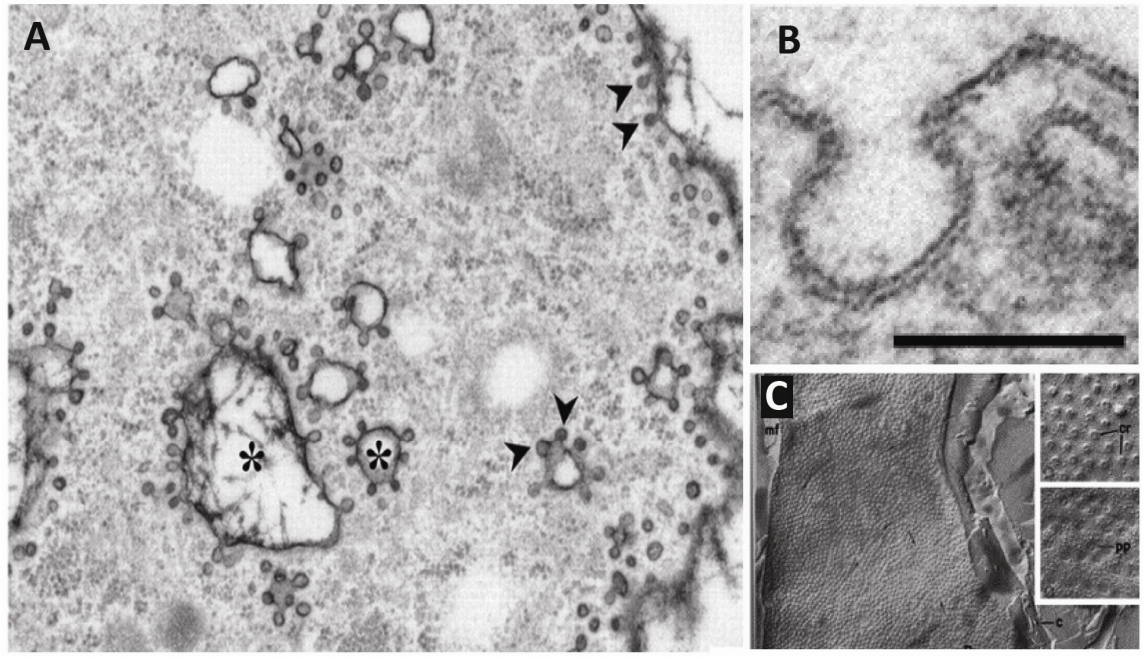


Figure 3: Morphology of caveolae. A. 3T3-L1 adipocyte labeled with an electron-dense marker to delineate the cell surface. Caveolae are indicated by an arrowhead, clustered caveolae are indicated by an asterisk. B. Caveolae as seen by transmission electron microscopy C. In heart endothelial cells caveolae are organized in linear arrays, insets show higher magnification. A. adapted from (Parton et al., 2006) B and C are adapted from (Stan, 2005)

### Morphology of caveolae

Although caveolae are commonly found as single invaginations on the plasma membrane, they can also appear as chains or grape-like clusters (Peters et al., 1985). The consequence of clustering of caveolae remains unknown. In muscle cells, caveolae can connect to the Transferse T-tubule system, a system of surface-connected membranes that conduct nerve impulses trough the muscle fiber. In these cells, besides being involved in endocytosis, caveolae are also involved in transmitting the nerve impulse (Ishikawa, 1968). In human fibroblasts, caveolae can be found in linear arrays in the plasma membrane (Rothberg et al., 1992) (figure 2). However, in

polarized cells, caveolae are mostly found at the basolateral surface (Verkade et al., 2000). In migrating endothelial cells, caveolae are not found throughout the plasma membrane, as they are mostly found in the retracting edge of the cell (Parat et al., 2003). The reason why caveolae are localized in this part of the cell during migration is discussed in chapter 7.

### Lipid rafts and caveolae

Lipid rafts are 'small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipid-enriched domains that are involved in several cellular processes (Pike, 2006). Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions (Pike, 2006). Rafts consist of cholesterol, sphingolipids (glycosphingolipids and sphingomyelin) and lipid modified-proteins such as glycosylphosphatidylinositol (GPI)-linked proteins (Simons and Toomre, 2000). Lipid rafts are found in the extracellular layer of the plasma membrane, however it is suggested that long fatty acyl chains of sphingolipids can span through both layers whereby they can interact with other lipids on the intracellular membrane (Rietveld and Simons, 1998). Caveolae share the same biochemical properties as rafts; both caveolae and rafts are resistant against mild nonionic detergents (e.g. Triton X-100 at 4°C) and can be purified by sucrose gradient ultracentrifugation, where they are found in the same fraction of detergent resistant fractions of cold lysates (Anderson, 1998). This suggests that caveolae contain the same components (lipids and proteins) as lipid rafts.

### Caveolae components

Caveolin is functionally the main component of caveolae (will be discussed in chapter 3) as it is required for the biogenesis of caveolae (Razani et al., 2001a). As stated before, caveolae do not have an electron-dense coat. However, proteins involved in formation of a caveolae, like caveolins and cavins, are often called members of the caveolar coat. Besides structural components such as cholesterol and sphingolipids, caveolae can also contain signaling proteins and growth factor receptors, such as Src family kinases (Sargiacomo et al., 1993), PDGF receptor (platelet derived growth factor) (Liu et al., 1996) and EGF receptor (epidermal growth factor) (Smart et al., 1995). Most of the proteins endocytosed by caveolae have a lipid modification such as GPI-linked proteins (for example the folate receptor) (Rothberg et al., 1990), or palmitoylation (caveolin) (Rothberg et al., 1992), or are transmembranes (MHC I) (Stang et al., 1997) or are able to bind to other structural caveolae proteins (like Src kinase and EGFR which are able to bind to caveolin) (Couet et al., 1997a, Smart et al., 1995). The best described components of caveolae are listed below in table 1 and their involvement in caveolae trafficking or biogenesis is discussed below.

Caveolae cargo

Caveolae are involved in endocytosis and transcytosis (Smart et al., 1999). They are responsible for the internalization of various lipids, ligands, receptors and pathogens. For example glycosphingolipids, GPI-anchored proteins (Brown and Rose, 1992), albumin (Schnitzer et al., 1994) and iron (Mineo and Anderson, 2001) (See table 1). Besides the nutrients and molecules that a cell needs to survive, also several pathogens use caveolae to enter the cell, like simian virus 40 (SV40) (Stang et al., 1997) and certain strains of *Escherichia coli* (Shin et al., 2000). They bind to transmembrane receptors (MHC class I and CD48 respectively) that are present in caveolae. Also bacteria toxins, like cholera and tetanus toxin, are able to use this endocytic pathway. Other molecules that have been suggested to be internalized via caveolae include the tetraspanin CD63 (Pols and Klumperman, 2009), calcium and iron (Mineo and Anderson, 2001). the EGF receptor is found in caveolar invaginations, can interact with caveolin and is internalized by clathrin-coated pits (Lamaze et al., 1993, Couet et al., 1997b). Upon EGF stimulation, the receptor moves away from the invagination (Mineo et al., 1999) Therefore it is likely that an activated EGF receptor cannot be internalized by caveolae. Integrin β1 (sometimes cross-linked with integrin α-2) is also reported to be internalized via caveolar endocytotis (Sharma et al., 2004). Caveolin-1 acts as a scaffold protein for several proteins (discussed below), via this binding it caveolae may internalize several proteins . Table 1 lists several molecules that can be endocytosed by caveolae.

Table 1: Overview of molecules that are involved in caveolae formation, trafficking and signaling, and an overview of several molecules that can be endocytosed by caveolae

| Class of molecule | Name of molecule | Component | Trafficking | Signaling | Cargo | Reference |
|---|---|---|---|---|---|---|
| **Lipids** | Cholesterol | + | | | + | (Smart et al., 1999) |
| | Glanglioside | + | | | + | (Smart et al., 1995) |
| | Sphingomyelin | + | | | + | (Brown and Rose, 1992) |
| **Proteins** | Caveolin (-1,2,3) | | + | | + | (Rothberg et al., 1992) |
| | Cavin (-1,2,3,4) | | + | | + | (Hill et al., 2008, Hansen et al., 2009) |
| | Dynamin | | + | | | (Henley et al., 1998) |
| | Filamin | | + | | | (Stahlhut and van Deurs, 2000) |
| **Membrane receptors** | EGFR | | | + | + | (Smart et al., 1995) |
| | PDGF receptor | | | + | + | (Liu et al., 1996) |
| | Angiotensin receptor | | | | + | (Wyse et al., 2003) |

| Class of molecule | Name of molecule | Component | Trafficking | Signaling | Cargo | Reference |
|---|---|---|---|---|---|---|
| | TGF-β receptor | | | + | + | (Di Guglielmo et al., 2003) |
| | Insulin receptor | | | + | + | (Cohen et al., 2003) |
| **Signaling molecules** | Src family kinases (Src, Fyn) | | + | + | + | (Cao et al., 2002) |
| | eNOS | | | + | + | (Feron et al., 1996) |
| | H-Ras | | | + | + | (Song et al., 1996a) |
| | G-proteins | | | + | + | (Li et al., 1995) |
| | Grb7 | | | + | + | (Lee et al., 2001) |
| **Trans-membrane** | CD63 | | | | + | (Pols and Klumperman, 2009) |
| | MHCI | | | | + | (Stang et al., 1997) |
| | CD48 | | | | + | (Shin et al., 2000) |
| **Integrins** | α5-β1 | | | + | | (Echarri and Del Pozo, 2006, Wary et al., 1998) |
| | β1 (α2β1) | | | | + | (Upla et al., 2004, Sharma et al., 2004) |
| **Ions** | Iron | | | | + | (Mineo and Anderson, 2001) |
| | Calcium | | | | + | (Mineo and Anderson, 2001) |
| **Pathogens** | SV40 | | | | + | (Stang et al., 1997) |
| | Cholera toxin | | | | + | (Torgersen et al., 2001) |
| | Tetanus toxin | | | | + | (Herreros et al., 2001) |
| | FimH expressing *E. coli* | | | | + | (Shin et al., 2000) |

Several proteins and lipids are involved in caveolae formation and function, although their precise mechanism of action remains to be established. Caveolae are carriers of several molecules. Not only nutrients can use this endocytic route, but also membrane receptors, membrane compartments and even pathogens do.

## 3. Caveolin proteins

Characteristics of caveolin protein

Caveolin is a 22 kDa molecule, which was first identified in 1992 as a component of the caveolae endocytosis machinery (Rothberg et al., 1992). Almost at the same time, an integral membrane protein of transport vesicles, VIP21 (Vesicular Integral-membrane Protein of 21 kDa), was cloned (Kurzchalia et al., 1992). This protein is involved in trafficking from the Golgi complex to the plasma membrane in MDCK (Madin Darby canine kidney) cells. The cDNA of caveolin and VIP21 is identical (Glenney, 1992), which suggests that caveolin/VIP21 not only plays a role in endocytosis, but also in trafficking from Golgi to the plasma membrane. Later, two other gene family members were discovered, named caveolin-2 and caveolin-3 (Scherer et al., 1996, Tang et al., 1996). Due to this discovery, the original caveolin is now termed caveolin-1. Caveolin-1 and caveolin-3 are highly homologous, with ~85% similarity and ~65% identity (Tang et al., 1996). The sequence of caveolin-2 differs from the other two, as it is only ~38% identical to caveolin-1 (Scherer et al., 1996). All three caveolins contain an identical amino-acid stretch of eight amino-acids, the 'FEDVIAEP' motif, which is called the 'caveolin signature motif' (Scherer et al., 1996). Although the sequences of caveolin-1 and caveolin-3 are highly homologous, the expression patterns of these proteins differ. Caveolin-1 and caveolin-2 are co-expressed in many cell types, the levels of expression varies between different cell types with the highest levels in endothelial cells, pneumocytes, adipocytes and fibroblasts (Scherer et al., 1994). Caveolin-3 is only expressed in muscle cells, e.g. skeletal, cardiac, and smooth muscle cells (Tang et al., 1996). Caveolin-3 is therefore also called M-Caveolin (Song et al., 1996b). It has been thought that expression of caveolin in muscle cells is restricted to caveolin-3 (Tang et al., 1996), however, low levels of caveolin-2 expression are also found in cardiac myocytes (Rybin et al., 2003). Recently, are in mouse and rat cardiac myocytes of the atria co-expression of caveolin-1 and caveolin-3 observed, but not in ventricles. This observation was made using RT-PCR and western blot analysis (Volonte et al., 2008).

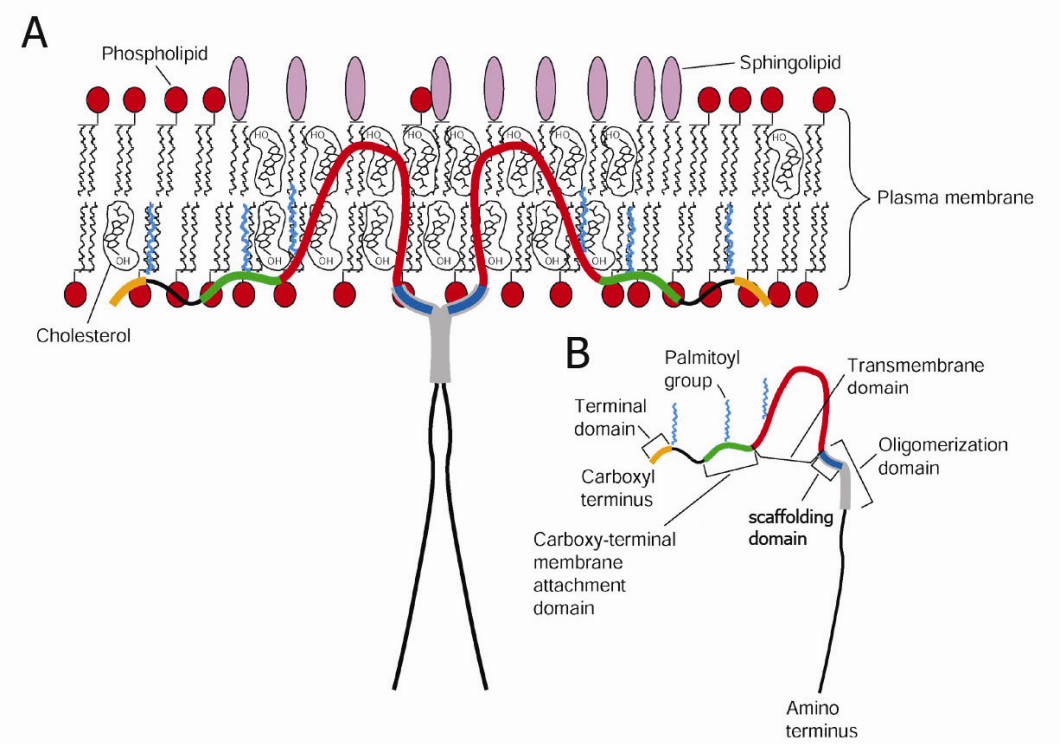


Figure 3: Structure and topology of caveolin-1. A. Caveolin proteins are localized in the membrane. Via the oligomerization domain they can form clusters of 14-16 monomers. The amino- and carboxylterminus are localized in the cytoplasm. B. Structure of caveolin. The different domains are indicated with different colors. Adapted from (Williams and Lisanti, 2004).

Structural features of caveolin

Caveolin is an integral membrane protein, with both the amino- and carboxy-terminus directed to the cytoplasm (figure 3) (Sargiacomo et al., 1995). Caveolin can be phosphorylated at tyrosine residue 14 by tyrosine kinases, like Src, Fyn and Abl. Phosphorylation of this residue will increase the induction of endocytosis, signal transduction, cell migration and mechanotransduction as SH-2 domain proteins are able to bind (Sanguinetti et al., 2003, Sanguinetti and Mastick, 2003). Phosphorylation of serine residue 80 (Ser80) will make the protein more soluble and caveolin-1 will be secreted in this form (Schlegel et al., 2001). This is primarily found in exocrine cell types, like the secretory cells of the pancreas and differentiating ostoblasts, where caveolin-1 is found in secretory vesicles (Schlegel et al., 2001). Caveolin can also be post-translationally modified by palmitoylation (Dietzen et al., 1995). This palmitoylation occurs on three cysteine residues (133, 143 and 156) that are located in the carboxyl-terminus of caveolin (Uittenbogaard and Smart, 2000). Residues 82-101 of the amino-terminus are called the caveolin scaffolding domain (CSD) and has the sequence DGIWKASFTTFTVTKYWFYR. This domain can bind to molecules containing the caveolin-1 binding motif, ZXZXXXXZ or ZXXXXZXXZ. Z stands for the amino acids Phenylalanine (F),

Tryptophan (W) or Tyrosine (Y). X stands for any amino acid (Williams and Lisanti, 2004). Several well-known signaling molecules bind to this domain, like Src family tyrosine kinases (Couet et al., 1997a), endothelial nitric oxide synthase (eNOS) (Garcia-Cardena et al., 1997), c-Neu, H-Ras and G-protein-coupled receptors (GPCRs)(Li et al., 1996). Several of these proteins will be discussed below in chapter 'Functional implications of caveolin-1 in cancer'. The scaffolding domain of caveolin-2 is not able to bind to these molecules due to differences in the sequences compared to caveolin-1 (Tang et al., 1997). Phosphorylation of tyrosine residue 19 and 27 of caveolin-2 will result in a binding place for SH-2 domain containing molecules, like phosphorylation of tyrosine residue 14 on caveolin-1 (Wang et al., 2004).

The amino-terminus of caveolins contains an oligomerization domain (Sargiacomo et al., 1995). Caveolin-1 and caveolin-3 can form an oligomeric complex of approximately 14-16 monomers, which is necessary for caveolae formation (Sargiacomo et al., 1995, Monier et al., 1996). Interestingly, caveolin-2 cannot oligomerize on its own, only together with caveolin-1 or caveolin-3 (Scherer et al., 1997). This may be due to differences in the sequence of the oligomerization domain of caveolin-2 compared with caveolin-1, as it is suggested that caveolin-2 will not bind to caveolin-1 with the oligomerization domain, but with the membrane spanning domain (Das et al., 1999) Caveolin-2 cannot form caveolae in the absence of caveolin-1 and will be retained in the Golgi complex as monomers or dimers (Parolini et al., 1999). Thus for plasma mebrane localization of caveolin-2 the other caveolins are required, depending on the cell type.

Caveolin sequences are found only in vertebrates (Fugu (vertebrate fish), Rat, Mouse, Dog, Bovine, Xenopus and Human) and in *Caenorhabditis elegans* (Williams and Lisanti, 2004). The sequence of the caveolin family found in *C. elegans* is related to the vertebrate family, however the distance between the two genes in a phylogenetic three is large compared to the caveolin genes of vertebrates. One of the two exons encoding caveolin-1 in *C. elegans* is homologous to mammalian caveolin. Tang et al. suggest that the mammalian caveolins are derived from this exon (Tang et al., 1997).

## 4. Caveolae/Caveolin trafficking

Exocytic trafficking of caveolin proteins

Newly synthesized caveolin is produced in the endoplasmatic reticulum (ER), as an integral membrane protein (Monier et al., 1995). Oligomerization of the proteins will take place in the ER, after which it is transported to the Golgi complex (Monier et al., 1996). In the Golgi complex, the cysteine residues of caveolin are palmitoylated (Parat and Fox, 2001), although this is not necessary for transport to the plasma membrane (Dietzen et al., 1995). When caveolin exits the Golgi complex the vesicles are detergent resistant, as they contain lipid raft domains, like glycolsphingolipids and cholesterol (Pol et al., 2005). The vesicles are carriers of several molecules and receptors, like the angiotensin and insulin receptors (Wyse et al., 2003, Cohen et al., 2003). How these vesicles are formed is not entirely clear, however it is known that lipid rafts can not only localize at the plasma membrane but also at internal membrane compartments, like the Golgi apparatus (Gkantiragas et al., 2001). It may be possible that caveolins are involved in the formation of a caveola together with compartments of a lipid raft and traffic in this composition to the plasma membrane. The exocytotic caveolae vesicles require syntaxin 6, to be transported from the Golgi complex to the plasmamembrane (Choudhury et al., 2006).

Caveolae formation

Caveolin can oligomerize with 14-16 monomers via its oligomerization domain, whereby a complex of caveolins is formed (Monier et al., 1996). These complexes together form a caveola. Pelkmans and Zerial calculated that one caveola approximately contains 144 caveolin proteins (Pelkmans and Zerial, 2005). It is suggested that cholesterol is needed to create the curve of the invagination, and it is estimated that 13 molecules of cholesterol are required to form the characteristic Ω-shape of caveolae (Parton et al., 2006). Caveolae can stay at the plasma membrane for a long time, therefore it was thought that they are immobile (Thomsen et al., 2002). However, internalization of caveolae can be induced by phosphorylation of tyrosine residue 14 of caveolin-1 by Src family kinases (Fyn, Src and Abl) (Cao et al., 2002), one of the tyrosine kinases that localizes at caveolae by binding to the scaffolding domain. In addition internalization requires GTP, as the GTPase dynamin is needed for fission of the vesicle from the plasma membrane (Henley et al., 1998). Internalization of caveolae can be inhibited by integrins (del Pozo et al., 2005). Activated integrins can sequester phosphorylated caveolin at focal adhesions whereby internationalization is blocked (Echarri and Del Pozo, 2006). Wary et al. showed that caveolin-1 co-immunoprecipitates with integrin β1, indicating that these proteins are in a complex in WI-38 cells (Wary et al., 1998). If caveolin-1 is purified from cells it is found in oligomeric complexes (Lisanti et al., 1994), suggesting that the phosphorylated

caveolin may also in the oligomeric form present at focal adhesions. Another possibility is that caveolin-1 is able to form oligomers during the purification. Summarizing, phosphorylation of tyrosine residue 14 is necessary for internalization of caveolae. The sequestering of phosphorylated caveolin at integrins inhibits this internalization.

Another protein involved in caveolae formation is cavin, also known as PTRF (Polymerase I and transcript release factor (Jansa et al., 1998). In a caveola, cavin and caveolin are present in a 1:1 ratio. Therefore it was suggested that cavin is a member of the caveolar coat (Hill et al., 2008). Recently, three other adaptor proteins have been identified, cavin-2, cavin-3 and cavin-4 (also known as SDPR, SRBC and MURC respectively) (McMahon et al., 2009), of which cavin-4 is only expressed in muscle cells (like caveolin-3). Knock-down of cavin-1 and cavin-2 reduces the expression level of caveolin-1 (Hansen et al., 2009). Cavin-3 has another effect, it reduces the budding of caveolin-1 vesicles of the plasma membrane (McMahon et al., 2009). Over-expression of cavin-2 will change the morphology of caveolae as it results in larger caveolar vesicles (Hansen et al., 2009). It is not clear whether caveolin-3 interacts with cavin-4, and if cavin-4 has in muscles cells the same function as the other cavins in other cell types (Nabi, 2009).

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In order to create an invagination the structure of actin has to be rearranged. Therefore filamentous actin is broken down upon caveolae invagination and new arrangements of monomeric actin are composed (called the actin patch). Due to this rearrangement, fission of the caveolae and the initiation to intracellular transport is achieved (Pelkmans et al., 2002). Caveolar vesicles are connected to the actin cytoskeleton. There are several proteins known that connect caveolae to the actin cytoskeleton, for example cortactin and filamin. Cortactin can bind to dynamin and actin (Sauvonnet et al., 2005), and via these connections, cortactin can regulate the actin dynamics upon endocytosis of dynamin-mediated vesicles. Filamin is found to interact directly with caveolin-1 (Stahlhut and van Deurs, 2000). As filamin is an actin binding protein, it may be possible that caveolin-1 containing vesicles are transported via actin to the microtubule network by binding to filamin 1 (figure 4).

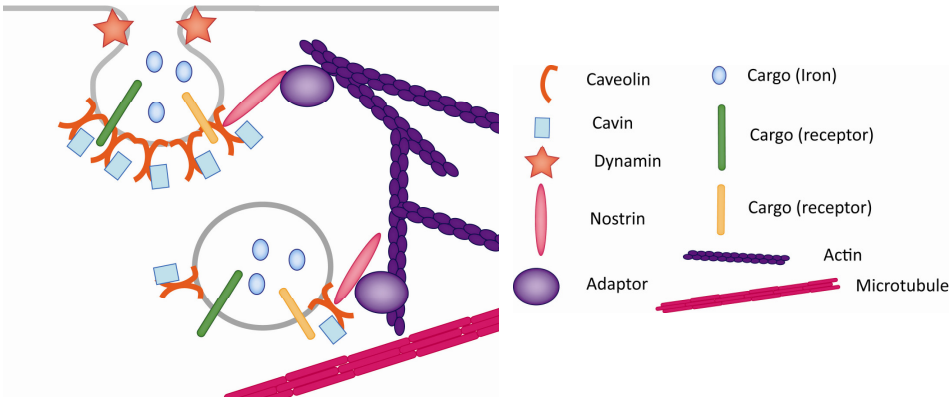


Figure 4: Schematic overview of proteins that are involved in caveolae-mediated endocytic transport. Growth receptors, like EGFR and molecules such as Iron can be endocytosed via caveolae. The fission of the invaginations is performed by dynamin. After the scission vesicles are transported via actin and microtubules to the caveosome. Adapted from (Siehoff-Icking, 2009)

Caveolae transport

When a caveolar vesicle is internalized, it is transported via microtubules. Loss of microtubules will lead to accumulation of caveolae vesicles at the plasma membrane (Tagawa et al., 2005). The speed of vesicle movement across the microtubules ranges from 0.3 to 2 μm per second. Organelles linked to the motor-proteins dynein and kinesin move with the same speed across microtubules, indicating that caveolar vesicles may also be transported via these motor-proteins (Mundy et al., 2002). The microtubules can bring the endocytic caveolar carriers to caveosomes, which are clustered in the proximity of the microtubule organizing center, shown in CHO cells (Mundy et al., 2002). This specialized, caveolin-rich and pH-neutral endosome is an intermediate organelle in caveolar uptake (Pelkmans et al., 2001). The idea that the caveolar endocytic pathway leads to a different organelle than the clathrin-mediated pathway, came by investigation of cholera toxin uptake (Orlandi and Fishman, 1998). It was found that the cholera toxin is toxic when it reaches the Golgi apparatus, however if endocytosis via caveolae is blocked the toxin will not be toxic for the cell. Interestingly, inhibition of clathrin-mediated endocytosis, only has a small effect on the toxicity (Orlandi and Fishman, 1998). This led to the suggestion that endocytosis of caveolar vesicles leads to another organelle than uptake of clathrin-coated vesicles does. Later Pelkmans et al. observed, that caveolae fuses with the caveosome by investigating the uptake of SV40 (Pelkmans et al., 2001). They found several caveosomes throughout the cytoplasm, with diverse shapes and sizes. The caveosome membrane consists of cholesterol and caveolin-1 which is present in the same form as on the plasma membrane. The pH of this organelle is neutral. The caveosome functions as a sorting compartment, for instance caveolin-1 is recycled back to the plasma membrane, cholera toxin is transported to the Golgi system and SV40 will be escorted to the ER (Kirkham et al., 2005). Recently it was found that another route can be taken from the caveosome as it leads to the

lysosome and protein degradation. This classic endocytic route was shown in HepG2 cells, where CD63 (late endosomal marker) and caveolin-1 accumulate in multi-vescular bodies upon endocytosis of albumin (Botos et al., 2008).

It is difficult to distinguish caveolar vesicles from the other parts of a cell, like lipid rafts, and most cargo proteins are endocytosed by several pathways, therefore it is difficult to investigate how the caveolar endocytic machinery works exactly. However the recent findings concerning components of the pathway and the identification of new players help solve the puzzle of caveolae internalization.

## 5. *In vivo* loss of caveolin

*In vivo*, the loss of caveolin was first assayed in *C. elegans* using RNA interference (Scheel et al., 1999). The only observed effect in these animals was an acceleration of the meiotic cell cycle, resulting in an increase in egglaying by hermaphrodites (Le Lay and Kurzchalia, 2005). Due to the mild effects of caveolin loss in *C. elegans,* caveolin-null mice were generated. Knock-out mice were created for all three caveolin proteins (Galbiati et al., 2001a, Drab et al., 2001, Razani et al., 2002b, Razani et al., 2001a). All mice were still viable, indicating that other pathways or the other caveolins might take over the role of caveolin (Razani et al., 2001a). Cells of caveolin-1 and caveolin-3 knock-out mice are not able to form caveolae in the tissues were the protein normally is expressed (Razani et al., 2001a, Drab et al., 2001). However, loss of caveolin-2 has no effect on caveolae formation (Razani et al., 2002b). Another phenotype demonstrated by caveolin-1 knock-out mice was hyperproliferation of cells in the lungs (Drab et al., 2001). Interestingly, loss of caveolin-2 also induces hyperproliferation in the lung, suggesting a role for caveolin-2 in these cells as well (Razani et al., 2002b). It might be possible that caveolin-2 regulates the functioning of caveolin-1, however this must be tissue-specific (lung), as other tissues (such as adipocytes) are only affected in caveolin-1 knock-out mice and not in caveolin-2 knock-out mice. The suggestion that caveolins have an important role in lung tissue is supported by the fact that approximately 70% of the plasma membrane of the alveolar septa is composed of caveolae (Gumbleton, 2001). Maybe the caveolae in the lung are necessary to have a response on shear stress (force on vessels). The lung is subjected to high shear stress when blood from the heart is pumped into the lung. A response to shear stress is mechanosensing. Caveolin-1 can be functional in mechanosensing, by activating the p42/44 pathway to induce cell proliferation as a reaction on shear stress (Park et al., 2000). There are suggestions that caveolin-2 may be also involved in the regulation of mechanosensing (Boyd et al., 2004), explaining the importance of caveolin-2 expression in lung tissue.

Caveolin-1 knock-out mice on a high fat diet stay leaner compared to wild type mice on the same diet (figure 5) (Razani et al., 2002a), suggesting that they are resistant to obesity. In normal adipocytes high expression levels of caveolin-1 were found. In these cells caveolin-1 is required for the transport of fatty acids from the plasma membrane to lipid droplets. In caveolin-1 knock-out mice, adipocytes showed smaller lipid droplets in fat pads (Razani et al., 2002a). The plasma levels of insulin, glucose and cholesterol of these mice were normal, suggesting that the intake of the amount of fat was not altered. The leaner phenotype is probably caused by the inability of these mice to convert triglycerides to a lipoprotein form, as increased levels of triglycerides were found in the plasma of the caveolin-1 knock-out mice (Razani et al., 2002a).

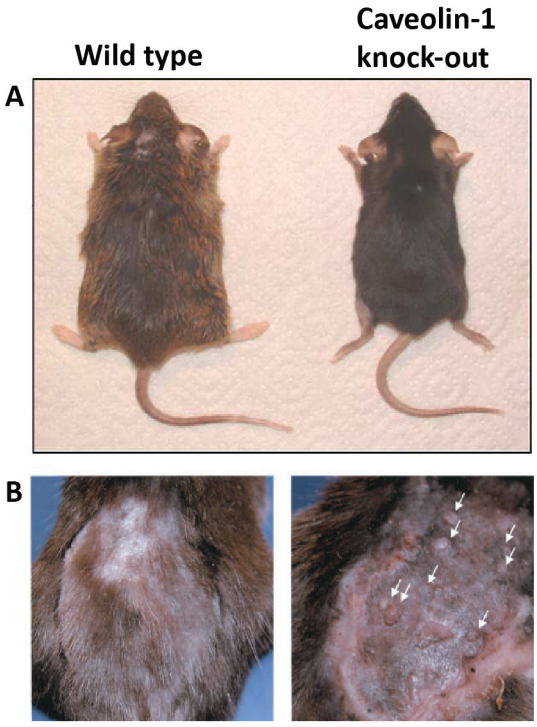


Figure 5: Phenotypes of caveolin null mice. A. Caveolin-1 knock-out mice on a high-fat diet stay leaner than wild type mice which were on the same diet. Photographs are taken after six months. B. Hypersensitivity of caveolin-1 knock-out mice to carcinogen induced epidermal tumors. Caveolin-1 knock-out mice develop tumors earlier after exposure (16 weeks) to the carcinogen DMBA (7,12-Dimethylbenz(a)anthracene) as wild type. A. Adapted from (Le Lay and Kurzchalia, 2005) B. Adapted from (Capozza et al., 2003)

Besides a loss of sarcolemmel (muscle cell plasma membrane) caveolae, loss of caveolin-3 induces myopathy of skeletal muscles, cardiac hypertrophy, loss of dystrophin-glycoprotein in lipid rafts and abnormalities in the T-tubule system (Galbiati et al., 2001a). These symptoms resemble the phenotype of patients with limb-girdle muscular dystrophy (LGMD-1C), this diseaese is related to a 95% decrease in caveolin-3 expression (Minetti et al., 1998).

An overall observation of caveolin knock-out mice was that they have a shorter life-span (Park et al., 2003), in contrast to the observation in *C. elegans*, were life span was not affected (Scheel et al., 1999). However this might be a result of secondary complications, such as the pulmonary hyperplasia or cardiac hypertrophy.

Tumorigenesis is correlated to a loss of caveolin-1 (Glenney and Zokas, 1989). Interestingly, caveolin-1 knock-out mice do not develop spontaneous tumors. However, when exposed to carcinogens, caveolin-1 knock-out mice show higher tumorigenicity compared to wild-type mice (figure 5) (Razani et al., 2001a, Capozza et al., 2003). Upon analysis, an increase in cyclin D and ERK1/2 level was observed in the epithelial tumors (Capozza et al., 2003). How caveolin and caveolae may be involved in cancer is discussed in chapter 6.

## 6. Caveolin and cancer

In cancer, changes can occur in the genetic code which alters the structure, expression or function of proteins, whereby signals can be interpreted incorrectly. The unique character of cancer cells is the consequence of mutations in specific genes (Hanahan and Weinberg, 2000). These genes encode proteins, which are involved in division, apoptosis, migration and how a cell can influence its environment. All these genes can be classified in to two groups; oncogenes and tumor suppressor genes (Hanahan and Weinberg, 2000). An oncogene is a gene that is known to contribute to tumorigenesis if the gene is mutated or the expression level is up-regulated. The protein where the oncogene encodes for is called an oncoprotein. A tumor suppressor gene is sometimes called an anti-oncogene. Tumor suppressor genes are genes which normally protect cells from transformation. By a mutation in a tumor suppressor gene the coding protein will lose its function and the cell can develop into a cancer cell (Hanahan and Weinberg, 2000). Loss of heterozygosity (LOH) is a process which often occurs in cancer. Loss of one functional copy of a tumor suppressor gene is often observed in cancer. However this does not lead to a cancer cell, as the other allele of the gene is still functioning normally (Cavenee et al., 1983). A second hit in the other allele is required to repress the tumor suppressor activity of the encoding protein and to contribute to the transformation of a normal cell into a cancer cell (Cavenee et al., 1983). One mutation in an oncogene or tumor suppressor gene is often not sufficient to change a cell in a cancer cell, mostly 5-7 mutations in different genes are required, although it depends on which gene is mutated and in which tissue this occurs (Hanahan and Weinberg, 2000).

Pathways involved in proliferation and cell survival are often mutated and up-regulated in cancer cells. Three important pathways in these processes are depicted in the figure below (figure 6), the p42/44 map kinase pathway (RAS/MEK/ERK), the PI3-kinase pathway (phosphatidylinositol 3-kinase) and the activation of EGF receptor upon EGF binding. Upon binding of EGF to the EGF receptor, the EGF receptor will dimerize with another EGF receptor. This dimerization will lead to an auto-cross-phorylation, resulting in activation of the kinase domain of the EGF receptor. This kinase domain can activate Grb2 an activator of the RAS/MEK/ERK pathway (Yamazaki et al., 2002).

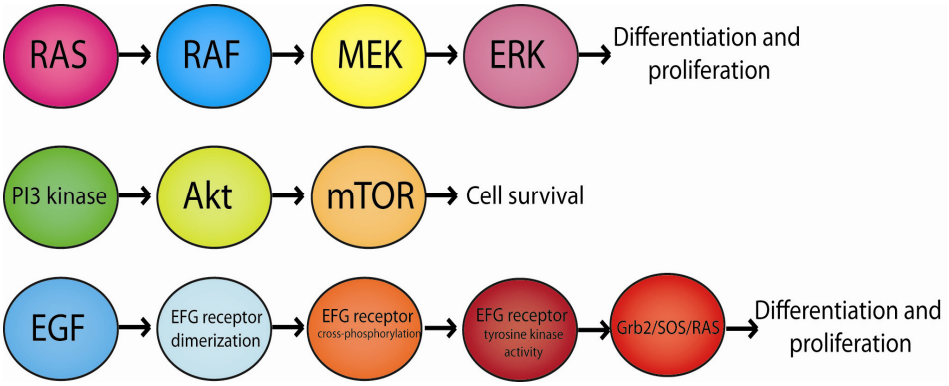


Figure 6: Schematic overview of three signaling pathways. The Ras pathway (also called the p42/44 pathway) is an important pathway in proliferation. PI3-kinase-Akt pathway is known to be an important pathway in cell survival. Epidermal growth factor (EGF) is a known growth factor. The EGF receptor has tyrosine kinase activity. Upon binding of the ligand, the receptor will dimerize and cross-phosphorylate the other receptor resulting in kinase activity of the receptor. The kinase will phosphorylate and thereby activate the Grb2/SoS/Ras pathway.

The first link between caveolin and cancer

Caveolin-1 was identified as a protein phosphorylated upon Rous sarcoma virus in chick embryo fibroblasts (Glenney and Zokas, 1989). Beside Gag, Pol and Env proteins, the virus also contains Src kinase, which phosphorylates caveolin-1 on a tyrosine residue in infected cells. Due to this observation, the presence and phosphorylation of caveolin-1 were associated with events involved in cell transformation. Also the observation that the level of caveolin-1 was reduced and there were no caveolae found at the plasma membrane in NIH-3T3 cell lines, which were transformed (v-Abl, bcr-Abl, Hras, polyoma virus mTag and CrkL oncogenes), (Koleske et al., 1995), contributed to the idea that caveolin-1 may be involved in cancer.

The CSD of caveolin can bind to many signaling proteins (see table 1). Many of these signaling proteins are known as proteins encoded by proto-oncogenes, such as the tyrosine kinase Src. By the binding of the signaling molecules, proteins are clustered at the plasma membrane. Due to this clustering, signaling can be enhanced. Caveolin-1 can also interact directly with many of these molecules and thereby influence the oncogenic activity of the proteins. An example of such direct interaction is the inhibition of heterotrimeric G-proteins by the CSD. This binding will inhibit GDP/GTP exchange, whereby the protein cannot be activated (Li et al., 1995). Heterotrimeric G-proteins are known proto-oncogenes as they can activate the Ras/MEK/ERK pathway (figure 6). It was found later that the CSD of caveolin-1 is involved in the negative regulation of other proto-oncogenic signaling proteins, like the autophosphorylation of Src kinase (Li et al., 1996), EGFR cross-autophosphorylation (figure 6) (Couet et al., 1997b), H-Ras (Engelman et al., 1997) and c-Neu (Engelman et al., 1998a). These proteins are all known to be

important players in cell transformation and tumor formation, as they are activators of several important pathways like the RAS/MEK/ERK, PI3kinase and JAK/STAT pathway. These pathways are involved in proliferation, survival and cell division. The signaling proteins which are summarized above, all have the same caveolin binding domain in their kinase domain, DVWSYGVTVWEL. Binding to this motif to caveolin-1 induces an inhibition of the kinase activity (Engelman et al., 1997). This suggests a general kinase inhibition activity for caveolin-1. The inhibiting role of caveolin on these proteins indicates that caveolin can be negatively involved in tumor formation.

Mutations in caveolin

Both caveolin-1 and caveolin-2 are localized on chromosome locus 7q31.1, near the often mutated D7S522 genetic marker (Engelman et al., 1998b). In several epithelial cancers (e.g. breast, ovarian, renal and prostate) mutations are found in the region around the D7S522 marker which is therefore known as a fragile region (named FRA7G), a region often affected in cancer. This D7S522 marker is also associated with loss of heterozygosity (Zenklusen et al., 1995). The closest known gene to this fragile region is caveolin-2, located 67 kb downstream of the D7S522 marker, caveolin-1 is located an additional 19 kb further downstream (Engelman et al., 1999). Caveolin-1 is probably one of the genes that is mutated around D7S522, as 16% of human breast cancers have mutations in caveolin-1 (Hayashi et al., 2001). The mutation identified in these human breast cancers was a mutation at codon 132, were a proline residue is replaced by a leucine (P132L). Caveolin-1 (P132L) has a different localization than wild-type caveolin-1 (shown in human mammary epithelial cells). The mutant is not able to traffic to the plasma membrane, as immunostaining shows caveolin-1 at the level of the Golgi complex (Lee et al., 2002). The P132L mutation behaves in a dominant-negative manner (one mutated allele is sufficient to have the effect of total loss of the gene) and not in a LOH manner as was suspected by its location on 7q31.1. This was concluded by the observation that in Cos-7 cells transfected with caveolin-1 (P132L), even when wild-type caveolin-1 is present in cells, both are not able to reach the plasma membrane, but remain in the Golgi apparatus (Lee et al., 2002). The dominant-negative action of the P132L mutation explains the observation in breast cancer patients where only one allele is mutated and caveolae were completely lost at the plasma membrane (Hayashi et al., 2001).

Another study showed that caveolin mutations only occur in ER-$\alpha$ (estrogen receptor) positive breast tumors, with an incidence of 35% and 0% in ER-$\alpha$ negative tumors (Li et al., 2006). The incidence of caveolin mutation in ER-$\alpha$ negative and positive breast tumor was 20% in this study (Li et al., 2006), comparable to the 16% of the previous study (Hayashi et al., 2001). Therefore, it is suggested that caveolin-1 is involved in the regulation of ER-$\alpha$ expression,

which is supported by the fact that ER-$\alpha$ is up-regulated in mammary cells of caveolin-1 knock-out mice, compared to wild-type mice (Li et al., 2006). How the expression level of ER-$\alpha$ is regulated by caveolin-1 is not known yet. Beside the P132L mutation, a C133R mutation in caveolin-1 was found in breast cancer patients (Hayashi et al., 2001). Later six other mutations in caveolin-1 were found in ER-$\alpha$ positive breast tumors (table 2) (Li et al., 2006). All eight mutations are located in or near the hydrophobic region of caveolin-1. Whether all mutations behave in a dominant-negative manner is not known yet (Li et al., 2006).

Table 2: Mutations of caveolin-1 found in human breast cancer.

| Amino-acid change | Mutation |
|---|---|
| Y118H | TAC - CAC |
| W128Stop | TGG - TAG |
| P132L | CCA - CTA |
| C133R | TGC - CGC |
| S136R | AGC - CGC |
| I141T | ATT - ACT |
| Y148S | TAT - TCT |
| Y148H | TAT - CCT |

All mutations are missense mutations in the hydrophobic region of caveolin-1, except the W128stop mutation, which induces an early stop.

Caveolin: a tumorsuppressor or oncogene?

Due to the down-regulation of caveolin-1 and the loss of caveolae at the plasma membrane upon transformation of NIH-3T3 cells (Koleske et al., 1995), it was suggested that caveolin may act as the protein product of a tumor suppressor gene. This was supported by the fact that tumor formation, with hyperactivation of the p42/44 MAP kinase pathway (RAS/MEK/ERK) was observed when NIH-3T3 fibroblasts with a down-regulated endogenous caveolin-1 were injected in nude mice (Galbiati et al., 1998). In addition, tumor cells are mostly undifferentiated cells, therefore the expression levels of caveolin-1 in differentiated cells and in undifferentiated cells were measured. It was observed that the expression of caveolin-1 is higher in differentiated cells (shown in adipocytes and Schwann cells) (Scherer et al., 1994, Mikol et al., 1999), suggesting again that down-regulation of caveolin-1 is important for tumor formation.

Confirmation of the tumor suppressor capabilities of caveolin was found in fibroblasts where the oncoprotein c-Myc can repress caveolin-1 expression (Park et al., 2001). Myc can activate pathways which are involved in cell survival (inhibition of apoptosis) and it is therefore known as a proto-oncogene. Up-regulation of Myc will contribute to tumor progression. As Myc is known to be a player in tumorigenesis, it is likely that genes down-regulated by Myc (such as caveolin-1) are tumor suppressor genes. In addition, the tumor suppressor effect was observed

in breast cancer cells. The breast cancer cell lines ZR75, MCF7 and MDA435 showed a lower expression level of caveolin-1 compared with normal epithelial breast cancer cell lines (Lee et al., 1998). If in these cells, expression of caveolin-1 was re-induced, the cells had a inhibition in growth compared with no expression of caveolin-1, shown in a soft agar colony formation assay (Lee et al., 1998), indicating a tumor suppressor phenotype upon caveolin-1 expression.

p53 is a key regulator in several pathways involved in cell survival and one of the best described tumor suppressor genes. Loss of p53 is often observed in tumors and mostly leads to induction of tumorigenesis (Jacks et al., 1994). Expression levels of caveolin-1 in mouse embryonic dermal fibroblasts (MEDF) isolated from p53 deficient mice and wild type mice were determined. The level of caveolin-1 was significantly decreased upon loss of p53 (Lee et al., 1998). These data are supported by the fact that the expression level of caveolin-1 in p53 knock-out mice is strongly reduced compared to wild type mice, shown in lung tissue (Williams et al., 2004). How loss p53 may regulate the down-regulation of caveolin-1 is not known yet.

Mammary hyperplasia with increased side branching was observed in caveolin-1 knock-out mice with a combined loss of the tumor suppressor gene INK4a, compared to mammary epithelial cell of INK4a$^{-/-}$ mice (figure 6) (Williams et al., 2004). In MEFs of these mice (Cav$^{-/-}$ INK4a$^{-/-}$) the loss of caveolin-1 resulted in a significant growth advantage and hyperactivation of the RAS/MEK/ERK kinase pathway like in the NIH-3T3 cells (Williams et al., 2004). The activation of the RAS/MEK/ERK pathway leads to more proliferation of the mammary epithelial cells (figure 7). An increase in cyclin D1 expression levels was also found, leading to faster cell division. These findings strongly suggest that caveolin-1 acts like a tumor suppressor gene, as loss of caveolin-1 in these cells will lead to more cell proliferation and more cell replication.

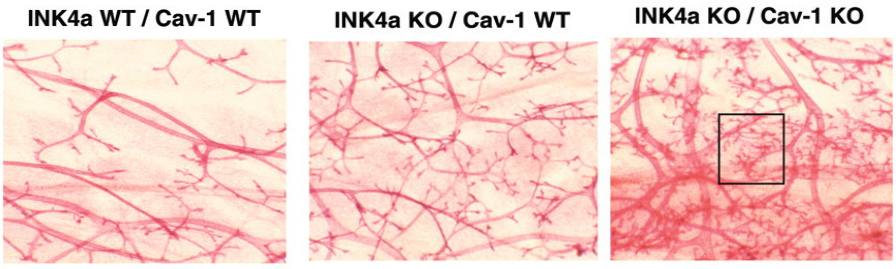


Figure 7: Mammary hyperplasia in Cav$^{-/-}$INK4a$^{-/-}$ mice. After interbreeding of Caveolin knock-out mice with INK4a knock-out mice, the generated double knock-out mice showed disturbed epithelial ductal morphology of the mammary gland. There was increased side-branching, hyperplasia and fibrosis, compared to wild type (Williams and Lisanti, 2005).

Caveolin-1 knock-out mice are more sensitive to chemical carcinogenic treatment as wild type mice as shown in figure 5 (Capozza et al., 2003). Epidermal tumors are developed earlier as response to this treatment by the caveolin-1 knock-out mouse. If the tumors were examined an up-regulation of the cell cycle checkpoint protein cyclin D1 and hyperactivation of the RAS/MEK/ERK pathway was found, similar to the $Cav^{-/-}INK4a^{-/-}$ mice.

However, there are also observations that caveolin-1 behaves like an oncogene. These observations were mostly done in prostate cancer. Increased levels of caveolin-1 is observed in patients with this type of cancer, 13% in well-differentiated tumors, 24% in moderately differentiated tumors and 39% of poorly differentiated tumors, respectively (Yang et al., 1999). In a mouse study, using the TRAMP (transgenic adenocarcinoma of mouse prostate) mouse model, it was shown that caveolin-1 expression is increased when compared to normal prostate epithelium (Williams et al., 2005). When TRAMP mice were interbred with caveolin-1 knock-out mice, the incidence of prostate tumors at 28 weeks of age was decreased and less metastases were found in the lymph nodes and distant organs (Williams et al., 2005). These results suggest that caveolin-1 may act as an oncogene in prostate cancer.

The different observations whether caveolin-1 acts like tumor suppressor gene or an oncogene can be explained by the posttranscriptional modification of caveolin-1. Upon phosphorylation on tyrosine residue 14 of caveolin-1 present on caveolae, internalization will be induced. However, phosphorylated caveolin-1 is often present at focal adhesions, bound to integrins. The phosphorylation on tyrosine 14 will inhibit the tumor suppressor function of the CSD, as phosphorylated caveolin-1 can bind to tyrosine kinases with an SH-2 adaptor domain, such as Grb7 and Src (Lee et al., 2001). Via binding of for example Grb7, caveolin-1 is able to activate the RAS/MEK/ERK pathway, leading to anchorage independent growth and cell proliferation (Lee et al., 2001).

The phosphorylation on serine residue 80 may also enhance the tumorigenic character of caveolin-1. If serine 80 is phosphorylated, caveolin-1 is not able anymore to maintain its positition in the membrane as an integral membrane protein and switches into a more soluble form (Schlegel et al., 2001). If the serine residue is mutated into an alanine (S80A) in MDCK cells, a mutation whereby phosphorylation is not possible anymore, no secreted caveolin-1 is found, while normally these cells do secrete caveolin-1 (Schlegel et al., 2001). Cells where the serine 80 residue is mutated into a glutamate residue, which will mimic phosphorylation, caveolin-1 is no longer found at the plasma membrane, but at the ER ready for secretion. Here caveolin-1 will be targeted to secreted vesicles (Schlegel et al., 2001). Soluble caveolin-1 is found

in the lumen of ER and not at the membrane whereby the scaffolding domain will not be able to perform its tumor suppressor activity.

The differences between posttranscriptional modifications like the phosphorylation on tyrosine 14 and serine 80 may be the cause of the different character of caveolin-1 in different tumor types and tumor stages. There are observations that prostate cancer cells secrete soluble caveolin-1. For example androgen-insensitive prostate cancer cell lines have a high expression level of soluble caveolin-1 (Tahir et al., 2001). It would be interesting to know whether these phosphorylations are absent in breast cancer and other cancer types.

## 7. Functional implication of caveolin-1 in cancer

Cancer cells regulate several processes differently than normal cells (Hanahan and Weinberg, 2000). A process which is often affected in cancer cells is the ability to go into apoptosis (programmed cell death). The cells evade apoptosis if the surrounding cells induce this process or if the cancer cell itself has DNA damage. Other capabilities of cancer cells are self-sufficiency in growth signals (often caused by an up-regulation of the Ras oncogene), insensitivity to anti-growth signals and thus the capability to replicate limitlessly (Hanahan and Weinberg, 2000). In which order cancer cells obtain these capabilities can vary between the different cancer types (Hanahan and Weinberg, 2000).

Due to the tumor suppressor capability of the scaffolding domain caveolin-1 may be involved in different processes required to transform a cell in a cancer cell. How caveolin-1 is involved in these processes is described below.

Role of caveolin-1 in signaling

Several signaling proteins are able to bind to the CSD of caveolin-1. Via this interaction, signaling proteins are able to remain and cluster at the plasma membrane. Signaling proteins can concentrate at the plasma membrane, as caveolar invaginations can steadily remain at the plasmamembrane and endocytosis of caveoale has to be induced by phosphorylation of caveolin. Due to the clustering of the signaling molecules on the plasma membrane an ideal situation for these proteins is created to have an increase in signaling transduction. However non-phosphorylated caveolin-1 is present at caveolae and the CSD of these caveolins is able to inhibit the activation of G-proteins, Src kinase and EGF receptor. For example, interaction between the EGF receptor and the CSD of caveolin-1, will inhibit the ability of the receptor to cross-phosphorylate the other EGF receptor (figure 6), whereby activation is inhibited (Couet et al., 1997b). It was later found that activation of PDGFR and TGF-β receptor was also suppressed if they were localized in caveolin-1-containing membrane domains (Matveev and Smart, 2002, Di Guglielmo et al., 2003). The CSD of caveolin-1 inhibits these growth factors by inhibition of the tyrosine kinase activity of the receptors. For example the TGF-β receptors (type I and II) colocalize with caveolin-1 positive compartments (Di Guglielmo et al., 2003). Normally receptor type I phosphorylates receptor type II, which activates the SMAD pathway. If caveolin-1 is overexpressed in fibroblasts, it was found that TGF-β signaling is decreased by inhibition of the SMAD pathway (Razani et al., 2001b). The SMAD pathway is involved in the transcription of growth factors. The CSD of caveolin-1 can interact with the TGF-β receptor type I, whereby its kinase activity is inhibited (Razani et al., 2001b). The negative regulation of caveolin-1 on the tyrosine kinase domain of a receptor (like TGF-β, EGFR and PDGFR) can be regulated via the binding of caveolin which stabilizes the kinase

compartment of the receptor in an inactive conformation. Another possibility is that dimerization of the receptor is inhibited (necessary for auto-phosphorylation of EGFR receptor and type I and II TGF-β receptor), whereby activation via transphosphorylation is inhibited. However, caveolin-1 can also negatively regulate the auto-activation of Src kinase, which does not need to form dimers for activation (Li et al., 1996), suggesting that another mechanism or multiple mechanisms are used by caveolin-1 for inhibition of kinases. Via the inhibition of tyrosine kinase receptors like the TGF-β receptor type I and EGFR, caveolin-1 inhibits growth factor pathways. One of the capabilities of a cancer cell is to be self-sufficient in growth factor signaling, and by inhibiting these growth receptor pathways caveolin-1 negatively regulates cancer cell transformation and thus contains tumor suppressor activity.

Interestingly, signaling by another tyrosine kinase receptor, the insulin receptor, is not inhibited by caveolin. Caveolar localization of the insulin receptor has been observed in adipocytes by some investigators (Kimura et al., 2002). If the expression level of caveolin-1 was increased in HEK-293T cells, which normally express extremely low levels of caveolin-1 (up to 40 times increased), no effect in the amount of insulin receptor present in the cells, or in the level of phosphorylation or in insulin receptor substrate 1 (IRS1) phosphorylation was observed (Wharton et al., 2005). This indicates that caveolin-1 is not able to regulate the activity of this tyrosine kinase receptor. It would be interesting to know which differences between EGFR and the insulin receptor are responsible for the different action caveolin-1 has on these receptor tyrosine kinases.

Apoptosis

Programmed cell death (apoptosis) is essential in development, immunology and homeostasis. One of the hallmarks of cancer cells is that they are mostly not sensitive to the induction of apoptosis (Hanahan and Weinberg, 2000). One of the best described pathways in cell survival is the PI3-kinase/Akt pathway (figure X). This pathway will lead to activation of the mTOR protein and subsequently to the inactivation of pro-apoptotic effectors like Bad and caspase-9 whereby apoptosis will be inhibited. Ceramide is known as one of the inducers of apoptosis, by inhibition of the PI3-kinase/Akt pathway. The precursor of ceramide (sphingomyelin) and the enzyme which is responsible for generating ceramide (sphingomyelinase) are present in caveolae in high levels (Liu and Anderson, 1995). Ceramide can regulate apoptosis by recruiting caveolin-1 to PI3-kinase complexes at the plasma membrane (Zundel et al., 2000). These complexes consist of activators of the PI3-kinase, such as PDGFR. Via inhibition of the tyrosine kinase activity of PDGFR caveolin-1 can negatively regulate the PI3-kinase/Akt pathway (Zundel et al., 2000). This is supported by the fact that over-expression of caveolin-1 in fibroblasts will induce death via a PI3-kinase

dependent pathway (Zundel et al., 2000). Cells transfected with wild type caveolin-1 induced apoptosis (chromatin condensation and plasma membrane permeability) upon apoptotic stimuli, while cells transfected with a mutant caveolin-1 missing the CSD survived this apoptotic trigger (Zundel et al., 2000). These results show that the scaffolding domain of caveolin-1 is negatively involved in the regulation of the PI3-kinase/Akt pathway and thus in the regulation of apoptosis. As cancer cells try to evade apoptosis, the induction of apoptosis of the CSD of caveolin-1 via the inhibition of PI3-kinase/Akt can be marked as a tumor suppressor activity.

Pro-apoptotic characteristics of caveolin-1 have been shown in MEFs (Galbiati et al., 2001b), NIH-3T3 fibroblasts and epithelial cells (T24 bladder carcinoma cells) (Liu et al., 2001). These cells became resistant to apoptosis when the expression of caveolin-1 is lacking. When caveolin-1 expression is restored, the cells become sensitive again to induction of apoptosis via Staurosporine exposure (Liu et al., 2001). Staurosporine is often used as an apoptosis inducer, it will increase the activity of caspase-3, an pro-apoptotic protein. The fact that cells which expressing caveolin-1 again were sensitive for this apoptotic inducer suggests that caveolin-1 normally is an activator of the apoptotic machinery.

Torres et al. found that caveolin-1 can regulate the expression level of survivin, a member of the inhibitor of apoptosis proteins (IAP) family (Torres et al., 2006). They suggest that this is regulated via a transcriptional mechanism (β-catenin/Tcf-Lef pathway), as the mRNA and protein levels of survivin were down-regulated upon re-expression of caveolin-1 in breast cancer cells (MCF-7 and ZR75). These cells normally have a reduced level of caveolin-1 (table 3). The promoter region of survivin consist of a Tcf-Lef binding site, β-catenin can activate the transcription factor Tcf-Lef whereby survivin will be transcribed. β-catenin and caveolin-1 can interact, and via this interaction is transcription via Tcf-Lef inhibited (Galbiati et al., 2000). Inhibition of this pathway would thus suggest a decrease in the expression level of survivin. As shown in the breast cancer cell lines and also in HEK-293T cells, expression of caveolin-1 will down-regulate the expression of survivin (Torres et al., 2006). Via a luciferase reporter assay was shown that the β-catenin transcription activity was decreased upon caveolin-1 expression, indicating that β-catenin inhibition is used for the decrease in survivin expression (Torres et al., 2006). Survivin is often present in high levels in cancer promoting cell survival, down-regulation of this protein demonstrates another tumor suppressor activity of caveolin-1.

In several cell lines also anti-apoptotic properties of caveolin-1 were found. In myeloma cells, caveolin-1 is found in a complex with IL-6 (interleukin-6) and IGF-1 (Insulin like growth factor). IL-6 and IGF-1 will then stimulate the activation of Src kinases, like Src and Fyn

(Podar et al., 2003). Src family kinases can phosphorylate caveolin-1 on tyrosine residue 14, via this phosphorylation step caveolin-1 is able to bind to SH-2 domain proteins like Grb7, leading to the activion of the survival pathway RAS/MEK/ERK (Lee et al., 2001). Hereby the oncogenic character of caveolin-1 is shown. In addition, apoptosis resistance increased in Rat1A cells and human prostate cancer cells (LNCaP) upon caveolin-1 over-expression (Timme et al., 2000). In the same cells it was shown that C-Myc induced apoptosis will lead to p53 activation and subsequent activation of the pro-apoptotic protein Bax. If caveolin-1 expression levels were elevated the LNCaP cells were less sensitive for OHT (4-hydroxytamoxifen), an inducer of c-myc induced apoptosis (Timme et al., 2000). In a later study the same group showed that co-expression of high levels of c-myc and caveolin-1 are a prognostic marker for human prostate cancinoma (104 patients tested) (Yang et al., 2005).

In addition, it was shown in mouse prostate cancer cells that caveolin-1 can inhibit the serine/threonine protein phosphatases, PP1 and PP2A (Li et al., 2003). PP1 and PP2A can regulate several important signal proteins, like Akt, Wee1 and PKA. The PI3-kinase/Akt pathway can be activated by PP1 and PP2A. The researchers claim that the inhibition of PP1 and PP2A are regulated via the scaffolding domain (Li et al., 2003), however the scaffolding domain is in other papers described as an inhibitor of the PI3-kinase/Akt pathway (Zundel et al., 2000). Whether phosphorylation of tyrosine residue 14 is required for this inhibition and thereby cell survival is not shown. This phosphorylation is known to be important to inactivate the tumor suppressor activity of the scaffolding domain and may clarify the cell survival activity of caveolin-1 in these cells.

Caveolin-1 can act as a pro- and anti-apoptotic protein. These differences are due to phosphorylation of tyrosine residue 14. This phosphorylation step will inhibit the tumor suppressor activity of the scaffolding domain of caveolin-1 whereby caveolin-1 can bind to SH-2 domain proteins, which are often well-known onco-proteins.

Cell cycle

Caveolin-1 expression is high in differentiated cells, these specialized cells do not replicate often, such as undifferentiated cells. Tumors can be graded by the degree of cellular differentiation. A tumor containing well differentiated cells, is classified as a low grade tumor and a tumor containing undifferentiated cells, is classified as a high grade tumor (Le Doussal et al., 1989). High grade tumors are more invasive, have high proliferation rates and are often metastatic. The high expression of caveolin-1 in differentiated cells suggests that caveolin-1 negatively regulates cell cycle progression. To examine this hypothesis the expression level of caveolin-1 during cell cycle progression in NIH-3T3 fibroblasts was measured. During the

$G_0/G_1$ phase the expression level of caveolin-1 was up-regulated compared to other phases, indicating that caveolin-1 is capable of repressing the induction of cell cycle progression (Galbiati et al., 2001b). If caveolin-1 expression was lost, a cell will start the cell cycle as a decrease in the number of cells in the $G_0/G_1$ phase was found upon this loss (Galbiati et al., 2001b). Over-expression of caveolin-1 in MEFs will result in an inhibition of proliferation and an increase of cells in the $G_0/G_1$ phase (Galbiati et al., 2001a). Inhibition of cell cycle progression can be induced by expression of the p53/p21 (WAF1/Cip1) pathway. Via a luciferase reporter assay was shown that caveolin-1 expression will activate p53, suggesting that the inhibition of cell cycle progression by caveolin-1 is p53/p21 (WAF1/Cip1) dependent (Galbiati et al., 2001b). One of the capabilities of cancer cells is that they can replicate limitlessly (Hanahan and Weinberg, 2000). The negative regulation of caveolin-1 on cell cycle progression contributes to the tumor suppressor character of caveolin-1.

The caveolin-1 knock-out mice also show evidence that caveolin-1 is involved in the negative regulation of cell cycle progression. The loss of caveolin-1 in these mice induces hyperplasia of the lung tissue (Razani et al., 2001a) and combined loss of the tumor suppressor INK4a, will induce hyperplasia of the mammary epithelium (figure 7) (Williams et al., 2004). Indicating that loss of caveolin-1 will induce cell proliferation, thus normally caveolin-1 will repress cell cycle progression. The combined loss of INK4a and caveolin-1 will lead to hyper-activation of the RAS/MEK/ERK pathway, which is involved in proliferation and cell cycle progression. In MEFs derived from caveolin-1/INK4a knock-out mice, a decrease of p21 (Cip1) was observed (Williams et al., 2004). p21 is a negative regulator of cell cycle progression, which is activated by p53, upon activation of p21 a cell will stay in $G_1$ phase. The decrease of p21 in the $Cav^{-/-}INK4a^{-/-}$ MEFs suggests that caveolin-1 regulates the cell cycle by p21 activation, as also described by Galbiati et al.

Cyclin D1 is a cell cycle checkpoint protein, elevated levels of this protein are necessary to progress from the $G_1$ phase to the S-phase in the cell cycle. In epithelial tumors derived from the $Cav^{-/-}INK4a^{-/-}$ mice, elevated levels of cyclin D1 were observed (Williams et al., 2004). This change in cyclin D1 levels can be explained by the fact that the cyclin D1 promoter is transcribed by Tcf-Lef proteins, like survivin (Torres et al., 2006). It may be possible that caveolin-1 is able to interact with Tcf-Lef like in the survivin regulation. This probably occurs via β-catenin and thereby regulate the transcription of cyclin D1 (Hulit et al., 2000). That caveolin-1 can regulate cyclin D1 expression was supported by the fact that over-expression of caveolin-1 in CHO cells reduced the transcription of cyclin D1 (Hulit et al., 2000), however expression of caveolin-1 had no effect if the Tcf-Lef binding site in the promoter of cyclin D1

was lost. The negative regulation of caveolin-1 on cyclin D1 will result in negative regulation of cell cycle progression.

Cell migration

Invasive tumors also have the ability to form tumors in other sites in the body. To form metastasis, cells need to migrate and enter a blood- or lymph vessel to traffic to another organ. Important regulators of cell migration are focal adhesions. Focal adhesions are large complexes of proteins linking the actin cytoskeleton to the extracellular matrix via integrins (Burridge et al., 1988). By dynamic regulation and localization of focal adhesions, cells are able to move through the matrix (Schoenwaelder and Burridge, 1999). Using monoclonal antibodies against phosphorylated caveolin-1 the localization of phosphorylated caveolin-1 at focal adhesions was confirmed (Swaney et al., 2006). Recently, it was shown that phosphorylated caveolin-1 here can stabilize FAK (focal adhesion kinase) (Joshi et al., 2008). Stabilization of FAK will lead to focal adhesion turnover, which is necessary for migration (Joshi et al., 2008).

In non-migrating endothelial cells, caveolin-1 is localized throughout the plasma membrane, which differs from migrating cells (Parat et al., 2003). Caveolae are only found at the rear edge of a migrating cell (Parat et al., 2003). Caveolin-1 is found in the rear of the migrating cells (like caveolae) upon two-dimensional movement, such as healing a wound in a monolayer (Parat et al., 2003). Interestingly, caveolin-1 localization differs from caveolae localization in cells which move through a pore (three-dimensional movement), as here caveolin-1 is found in the front of the cell (Parat et al., 2003). Interestingly, there is a difference in the localization of phosphorylated and non-phosphorylated caveolin-1. Phosphorylated caveolin-1 moves to the front of a cell, whereby the cell is polarized (Beardsley et al., 2005). The polarization may lead to directional migration. Directional cell migration was lost upon knock-down of caveolin-1. The phenotype could be rescued upon re-expression of caveolin-1 (Beardsley et al., 2005), suggesting that caveolin-1 is necessary for directional migration. Regulation of the polarization of caveolin-1 is regulated by amino residue 47 till 56. Sun et al. identified these 10 amino-acid residues located in the N-terminus of caveolin-1 that are required for the localization of caveolin. If these residues were replaced by alanine, caveolin-1 is found throughout the cell instead of in the rear of the cell (Sun et al., 2007). The sequence of these amino acids (47 – 56) are identical between caveolin-1 and caveolin-3 (Sun et al., 2007), suggesting that localization of caveolin-3 during migration of muscle cells is regulated at the same way as caveolin-1.

Angiogenesis

In cancer cells, migration is not only important for the formation of metastases, migration is also required for the movement of endothelial cells to form new blood vessels (angiogenesis). Upon exogenous stimulation of angiogenesis (with basic fibroblast growth factor) in caveolin-1 knock-out mice, an impaired angiogenic response was observed (Woodman et al., 2003). Also stimulation with matrigel and injection of a melanoma cell line (B16-F10) in the caveolin-1 knock-out mice gave a reduction in blood vessel formation (Woodman et al., 2003). In addition, the caveolin-1 knock-out mice showed a delay in cutaneous wound healing (Grande-Garcia et al., 2007), a process which besides proliferation is also dependent on migration. Suggesting that caveolin-1 is a player in the regulation of cell migration necessary for angiogenesis.

Caveolin-1 is involved in the regulation of several cellular processes, described above. Often is the function of the non-phosphorylated form of caveolin-1 examined, resulting in the conclusion that caveolin-1 acts as a tumor suppressor via the activity of the scaffolding domain. In some cases is the function of phosphorylated caveolin-1 (Y14caveolin) investigated, like the anti-apoptotic effect of Y14caveolin in myeloma cells (Podar et al., 2003). In these cases caveolin-1 stimulates tumor progression.

**Discussion**

Endocytosis is used by cells to absorb molecules from outside the cell, to internalize proteins present at the plasma membrane and to regulate signaling. One of the endocytic routes is mediated by caveolae. Caveolae are present in microdomains of the plasma membrane, lipid rafts (Palade, 1953). Caveolin is the main component of caveolae (Rothberg et al., 1992). It becomes increasingly clear that caveolin is not just involved in caveolae formation and endocytosis, but has a more diverse role in cellular functioning. Caveolin is not only involved in endocytosis, but also in the regulation of signaling, as many signaling molecules can bind to the scaffolding domain of caveolin and others to phosphorylated caveolin (Y14). Since the late 1980's it has been suggested that caveolin is involved in cancer (Glenney and Zokas, 1989). This thesis describes how caveolin-1 is involved in the different processes that drive tumorigenesis, such as apoptosis, cell proliferation and migration.

Expression of caveolin-1 and caveolin-2 is found in numerous cell types, with high levels being found in adipocytes, endothelial cells and fibroblasts (Razani and Lisanti, 2001). Expression of caveolin-3 is only found in muscle specific cells, like skeletal muscle, cardiac cells and in the diaphragm (Song et al., 1996b). Whether caveolin-1 and caveolin-2 expression is completely missing in muscle cells is still questionable. Co-expression of caveolin-1 and caveolin-3 is observed by Volonte et al. in atria cardiac myocytes, but not in ventrical cells. Expression of caveolin-1 in cardiac muscle cells would explain the cardiac defects which are observed in caveolin-1 knock-out mice (Razani et al., 2001a), however the heart also exists of non-muscle cells that could have caused the cardiac defects. The level of expression of caveolin-1 in the atria cardiac myocytes is not described yet. But if caveolin-1 would be present in low levels it would indicate that caveolin-3 is the most important caveolin in muscle cells and clarify why muscle cells contain a specific caveolin. From caveolin-3 knock-out mice is known that the level of caveolin-1 expression is not sufficient to form caveolae in muscles cells as they lack caveolae in muscle cells (shown in skeletal muscle cells) (Galbiati et al., 2001a) .

Caveolin knock-out mice of the three different caveolins are all viable (Razani et al., 2001a, Galbiati et al., 2001a, Razani et al., 2002b). It can be suggested that maybe one of the other caveolins may be able to take over the function of the others. However caveolin-1 and caveolin-2 are expressed in other cell types than caveolin-3 (Song et al., 1996b) and caveolin-1 -3 double knock-out mice are still viable (Park et al., 2002). The suggestion that caveolin-2 may compensate for the functions of caveolin-1 is not likely. Caveolin-2 is not very homologous with caveolin-1 and -3 (Tang et al., 1996). The scaffolding domain of caveolin-2

is therefore not able to bind to the same signaling molecules (Couet et al., 1997a). More importantly, caveolin-2 is not capable to form caveolae if caveolin-1 or -3 are not expressed (Razani et al., 2001a). Therefore it is clear that the caveolin proteins are not completely redundant. More likely it would be that another endocytic pathway will internalize molecules which normally are transported via caveolae.

Based on the tumor suppressor activity of the scaffolding domain of caveolin-1, low levels of caveolin-1 would be expected in cancer. However the level of caveolin-1 expression differs between different tumor types. In table 3, several cancer types are listed and whether caveolin-1 is up- or down-regulated is specified.

Table 3: Expression level of caveolin-1 in several cancer types.

| Cancer type | Expression level caveolin-1 | References |
|---|---|---|
| Urinary bladder | ↑ | (Fong et al., 2003, Rajjayabun et al., 2001) |
| Breast | ↓ | (Chen et al., 2004, Sagara et al., 2004) |
| Cervical | ↓ | (Chan et al., 2003) |
| Colon | ↑↓ | (Kim et al., 2006, Bender et al., 2000) |
| Lung | ↓ | (Liu et al., 2008, Belanger et al., 2004) |
| Melanoma | ↑ | (Podar et al., 2003) |
| Nasopharynchal | ↑ | (Du et al., 2009) |
| Ovary | ↓ | (Bagnoli et al., 2000) |
| Liver | ↑ | (Cokakli et al., 2009) |
| Prostate | ↑ | (Cui et al., 2001, Yang et al., 1998, Mouraviev et al., 2002, Bachmann et al., 2008, Tahir et al., 2001) |
| Renal | ↑ | (Horiguchi et al., 2004, Tamaskar et al., 2007) |
| T-cell leukemia | ↑ | (Hatanaka et al., 1998) |

The expression level of caveolin-1 between different cancer types differs. Up-regulation of caveolin-1 is best described in prostate cancer, where it may act as a tumor suppressor gene. Controversly, the expression of caveolin-1in breast cancer is down-regulated, indicating it may act as an oncogene.

The differences between the expression levels can be clarified by the fact that phosphorylation of tyrosine residue 14 of caveolin-1 can transform caveolin-1 from a tumor suppressor into an oncoprotein. Via this phosphorylation tyrosine kinases with an SH-2 adaptor domain are able to bind to caveolin-1 (Lee et al., 2001). Examples of these proteins are Grb7 and Src, both components of pathways which are often up-regulated in cancer. Another possibility is the phosphorylation of serine residue 80 of caveolin-1. Caveolin-1 will be more soluble upon this phosphorylation (Schlegel et al., 2001). Caveolin-1 phosphorylated on residue 80 is found to be secreted by specialized secretory cells (Schlegel et al., 2001). Soluble caveolin-1 has been found in high levels in prostate cancer (Tahir et al., 2001). It

would be interesting to know, whether caveolin-1 is phosphorylated in other cancer types with an up-regulation of caveolin-1.

Interestingly, there are different observations about the expression level of caveolin-1 in colon cancer. It is observed that the level can be increased (Kim et al., 2006), however a down-regulation of the expression level of caveolin-1 is also observed (Bender et al., 2000). A possible explanation could be that the expression level of caveolin-1 changes as the tumor progresses (Bender et al., 2000). Caveolin-1 expression is high in terminally differentiated cells, such as adipocytes and endothelial cells, where cell proliferation is inhibited (Scherer et al., 1994). To induce tumor formation, rapid proliferation is required and therefore down-regulation of caveolin-1 expression may be necessary. There are reports that the level of caveolin-1 is related to the invasiveness of the tumor (Cokakli et al., 2009). The higher expression level of caveolin-1 in these stages (phosphorylated or not) may be required for cell survival, migration and limitless replication (Ravid et al., 2006). A study providing information on tumor development and caveolin-1 expression at different stages and whether caveolin-1 is phosphorylated on residue tyrosine 14 or serine 80 would be very usefull to unravel the role of caveolin-1 expression during tumorigenesis.

At this time, there are eight point mutations known in caveolin-1 (table 2). However, only the P132L mutation is well described and often found in cancer (Hayashi et al., 2001). All mutations occur in a range of 34 amino acids, and in or near the transmembrane domain (Li et al., 2006). Whether these mutations result in the same phenotype as the P132L mutation would be interesting to know. The effect of the P132L mutation is that all caveolin-1 proteins (mutated or not) are stranded in the Golgi complex. This can be due to the difference in hydropathy (whether an amino acid is hydrophobic or hydrophilic). Some amino acids are hydrophobic (like isoleucine and valine) and others are hydrophilic (like arginine and lysine) (Kyte and Doolittle, 1982). The P132L mutation will induce a change from the hydrophilic proline to the hydrophobic leucine. This change can be responsible for the stranding at the Golgi complex of caveolin-1. Three of the other seven mutations will also result in a large change in hydropathy. The C133R mutation will induce a change from a hydrophobic amino acid into a very hydrophilic. S136R will give a change from a little hydrophilic to very hydrophilic and I141T will induce a change from very hydrophobic to hydrophilic. All these four mutation (P132L, C133R, S136R and I141T) give thus a difference in hydropathy, therefore they may have the same impact as the P132L mutation has.

The other mutations involve also changes whereby extra or less phosphorylation sites on caveolin-1 are created. In eukaryotic cells phosphorylation mostly occurs on serine (S),

threonine (T) and tyrosine (Y) residues. Three of the eight known mutations will change one of these amino acids (S, T or Y) into another amino acid. One of these (Y148S) will change a tyrosine residue into a serine, which will not change the capability to be phosphorylated, however kinases are often capable to phosphorylate a serine or threonine residue or capable to phosphorylate a tyrosine residue, so there will be no difference if the targeting kinase cannot phosphorylate both. Due to the two mutations (Y118H and Y148H) caveolin-1 cannot be phosphorylated at these locations anymore. The I141T mutation will create an extra phosphorylation site at caveolin-1. Whether this mutated site is phosphorylated in some cancer types is not known yet.
The tumor suppressor function of the scaffolding domain of caveolin may be taken over by other proteins as the caveolin knock-out mice not show spontaneous tumors (Razani et al., 2001a, Drab et al., 2001). However, if they are exposed to carcinogens, tumors will develop earlier in caveolin-1 knock-out mice compared to wild-type mice, indicating that caveolin-1 has an influence on tumorigenesis, as it may lower the threshold of tumor initiation. In tumor formation are often more mutations necessary (Hanahan and Weinberg, 2000). Loss of additional proteins due to the carcinogens may thus lead earlier to tumor initiation if caveolin-1 is lost, indicating that caveolin-1 in these epithelial cells normally functions as a tumor suppressor.

It would be interesting if one of the eight known mutations or another mutation would not sequester the protein in the Golgi complex, but has an effect on the inhibition of the CSD on Src kinase and EGFR. The CSD can inhibit oncogenic signaling by inhibiting for example Src (Li et al., 1996), thus a mutation in the CSD may result in an inactive CSD whereby the inhibition is relieved and caveolin-1 thus loses its tumor suppressor capacity. Normally signaling molecules are clustered at caveolae by caveolin-1, this clustering can lead to enhanced signaling and caveolin-1 will be a positive regulator of tumorigenesis.

A Japanese study showed that 16% of the breast cancer cases contain a P132L mutation in caveolin-1 (Hayashi et al., 2001). In a small American study (55 patients) 20% of all the breast cancer cases were shown to contain this mutation (Li et al., 2006). However a large study in the western world was never performed. It may be relevant to perform such a study, as cancer is one of the major causes of death in the western world (CBS). If such a study would confirm these percentages, caveolin-1 can be identified as one of the major players in breast cancer progression.

Screening for one of the eight mutations of caveolin-1 in breast cancer samples may result in earlier diagnosis of an ER-$\alpha$ positive tumor. As was shown that these mutations occur only in

ER-$\alpha$ positive tumors (35%), and ER-$\alpha$ expression is up-regulated in caveolin-1 knock-out mice (Li et al., 2006). The inactivation of caveolin-1 leads to increased ER-$\alpha$ in cultured cells (Li et al., 2006), suggesting that loss of caveolin-1 occurs before ER-$\alpha$ will be up-regulated and that caveolin-1 may even be involved in the up-regulation of ER-$\alpha$ (Mercier et al., 2009). If breast cancer patients are screened for loss of caveolin-1, therapy can be adjusted to these patients, as ER-$\alpha$ positive tumors can be treated by hormone therapy and ER-$\alpha$ negative tumors cannot.

A way how caveolin-1 may be used for clinical use in the future may be by designing a peptide containing the CSD sequence and a lipid modification whereby it will be anchored in the plasmamembrane. The tumor suppressor properties of the CSD, like sequestering of proto-oncogenic molecules at the plasma membrane and inhibit them, can be used to suppress tumor formation. Calbiochem® already made a peptide of the caveolin-1 scaffolding domain peptide (residue 82-101) fused to the N-terminus of the cell-permeable Antennepedia internalization sequence. The peptide does not contain residue 14 and 80 of caveolin-1 whereby phosphorylation of these residues is not possible and the peptide will only function as a tumor suppressor. The peptide of Calbiochem® can used to study the tumor suppressor activity of the CSD of caveolin1 *in vitro* and *in vivo*.

In summary, caveolin can act as a tumor suppressor gene and as an oncogene, depending on the phosphorylation status of the protein. The protein is involved in many processes which are hallmarks of cancer. More research will be necessary to understand the function of caveolin and caveolae during tumor initiation and progression.

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