ROLE OF CAVEOLIN-1 IN BROWN ADIPOSE TISSUE

Charlotte L. Mattsson
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Picture on cover: Cryosectioned brown adipose tissue (10 μm) stained with caveolin-1 (red), the lipid marker bodipy (green) and the nuclei marker Hoechst (blue). The image was acquired in a Zeiss LSM 510 Confocal Microscope.
“Knowing others is wisdom, knowing yourself is enlightenment”

Lao Tzu

“No amount of experimentation can ever prove me right; a single experiment can prove me wrong”

Albert Einstein

To all my loved ones
ABSTRACT

Caveolae are 50-100 nm invaginations in the plasma membrane. Caveolae and the protein caveolin-1 (Cav1) have been shown to be important in many signaling pathways in different cell types; however, in some cell types caveolae and Cav1 do not seem to affect the investigated signaling pathways. In my thesis, I have investigated the role of caveolin-1 (Cav1) in metabolism and β3-adrenergic, LPA-, EGF- and PDGF-receptor signaling in brown adipocytes.

Brown adipose tissue is responsible for nonshivering thermogenesis. Recent studies have shown that not only infants but also adult man can have brown adipose tissue and that the presence is negatively correlated with both obesity and age. By understanding how signaling for proliferation and differentiation in brown adipocytes is regulated, it could be possible in the future to activate brown adipose tissue to combat obesity and the metabolic syndrome.

In brown adipocytes, both epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) were able to induce proliferation, which was dependent on Erk1/2 activation. However, EGF and PDGF utilized different pathways to activate Erk1/2, with EGF signaling partially occurring via a Src-pathway (not involving PI3K/PKC) and PDGF via a PI3K/PKC/Src-pathway. Furthermore, LPA receptors were able to activate Erk1/2 via two pathways, one G_i/PKC/Src-pathway and one PI3K-pathway. For these receptors, Cav1-ablation did not affect the agonist-induced Erk1/2 activation. Cav1 was, however, required for proper β3-adrenergic receptor (β3-AR) signaling to cAMP and for adenylyl cyclase activity.

In Cav1-ablated mice, the adrenergic receptors are desensitized. However, this desensitization could be overcome physiologically, and the Cav1-ablated mice were therefore able to survive in prolonged cold by nonshivering thermogenesis.

In conclusion, ablation of Cav1 affected certain signaling pathways in brown adipocytes, while other pathways were not affected or could be physiologically rescued.
This thesis is based on the following papers, which are referred to in the text by their Roman numerals, respectively

I  Mattsson, C.L., Csikasz, R.I., Shabalina, I.G., Nedergaard, J., Cannon, B.
Caveolin-1-ablated mice survive cold by nonshivering thermogenesis, despite desensitized adrenergic receptors.
Submitted

II  Holmström, T.E., Mattsson, C.L., Fälting, J.M., Nedergaard, J.
Differential signaling pathways for EGF versus PDGF activation of Erk1/2 MAP kinase and cell proliferation in brown pre-adipocytes.
Experimental Cell Research, 2008, 314, 3581-3592

III Holmström, T.E., Mattsson, C.L., Wang, Y., Iakovleva, I., Petrovic, N., Nedergaard, J.
Non-transactivational, dual pathways for LPA-induced Erk1/2 activation in primary cultures of brown pre-adipocytes.
Submitted

IV  Mattsson, C.L., Andersson, E.R., Nedergaard, J.
Differential involvement of caveolin-1 in brown adipocyte signaling: impaired β3-adrenergic but unaffected LPA, PDGF and EGF receptor signaling
Submitted
Abbreviations

AC  Adenylyl cyclase
AR  Adrenergic receptor
ATGL  Adipose triglyceride lipase
BAT  Brown adipose tissue
Cav  Caveolin
cAMP  Adenosine 3’,5’-cyclic monophosphate
CSD  Caveolin scaffolding domain
DAG  Diacylglycerol
EGFR  Epidermal growth factor receptor
Erk  Extracellular-regulated protein kinase
FFA  Free fatty acid
G Battery text: inhibitory G-protein
GPCR  G-protein coupled receptor
Gs  Stimulatory G-protein
HSL  Hormone-sensitive lipase
IP3  Inositol triphosphate
LPAxR  Lysophosphatidic acid receptor x
MAPK  Mitogen-activated protein kinase
mβCD  Methyl-β-cyclodextrin
MEF  Mouse embryonic fibroblast
MEK  MAP/Erk kinase
mRNA  Messenger ribonucleic acid
MRI  Magnetic resonance imaging
NE  Norepinephrine
PDGFR  Platelet-derived growth factor receptor
PI3K  Phosphatidylinositol-3-kinase
PKA, PKB, PKC  Protein kinase A, B and C
PLA, PLC, PLD  Phospholipase A, C and D
PPAR  Peroxisome proliferator-activated receptor
PTRF  Polymerase I and transcript release factor
RTK  Receptor tyrosine kinase
siRNA  Small interfering ribonucleic acid
WAT  White adipose tissue
UCP1  Uncoupling protein 1
1. Introduction

For many decades, the fluid mosaic model proposed by Singer and Nicolson in 1972 was the basis for the understanding of the plasma membrane. In this model, proteins were floating around in the lipid membrane and the plasma membrane was regarded as being homogeneous (Singer and Nicolson 1972, Thomas and Smart 2008). However, work during the two last decades has changed this view of the plasma membrane. The plasma membrane is now known to contain both so-called liquid-disordered phases (liquid crystalline $l_c$ phase) and liquid-ordered phases ($l_o$) (de Laurentiis et al. 2007). These liquid-ordered phases, or microdomains, were named lipid rafts based on the different lipid composition they contain (mixtures of phospholipids, sphingolipids and cholesterol) compared to the rest of the plasma membrane. In addition to lipid rafts, caveolae were also discovered (Thomas and Smart 2008).

It has been proposed that lipid rafts instead should be called membrane rafts, with the definition “small (10-200 nm) heterogeneous, highly dynamic, sterol-and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions” (Pike 2006).

In this thesis, lipid rafts and caveolae together will be called membrane rafts (or written as caveolae/lipid raft), which are separate from the rest of the plasma membrane (which is called the bulk membrane). One major difference between lipid rafts and caveolae is that lipid rafts are flat on the plasma membrane while caveolae are invaginations in the plasma membrane. Since the lipid rafts are flat and cannot be detected by electron microscopy (as caveolae can be), they are harder to locate (de Laurentiis et al. 2007), and Munro wrote about them as being either “elusive or illusive” (Munro 2003). Usually the estimated diameter of the lipid rafts is 70 nm (but can be as little as 26 nm) and for caveolae, the estimated diameter is 50-100 nm (de Laurentiis et al. 2007).

In this thesis, caveolae and their components (section 2), and the model system brown adipose tissue (section 3) will be described. Caveolae have been proposed to be both metabolic and signaling platforms (de Laurentiis et al. 2007, Ortegren et al. 2007) and this will be discussed in section 4 (with regard to adipocytes) and in section 5, respectively.
2. Caveolae and caveolin

Already in the 1950’s caveolae were described by electron microscopy by Palade and Yamada (Palade 1953, Yamada 1955, Thomas and Smart 2008). Since then numerous studies have investigated caveolae and their importance in cellular processes, for review, see (Anderson 1998, Smart et al. 1999, Razani et al. 2002b, Quest et al. 2004, de Laurentiis et al. 2007, Parton and Simons 2007, Thomas and Smart 2008, Patel et al. 2008a, Patel et al. 2008b). Caveolae have been implicated in cell processes such as endocytosis, exocytosis, cholesterol homeostasis and signal transduction (Thomas and Smart 2008).

Many cell types have caveolae, and caveolae are highly abundant in adipocytes, fibroblasts, endothelial cells and certain muscle cells (Razani et al. 2002b, Thorn et al. 2003). However, in erythrocytes, lymphocytes and neurons, no caveolae have been found (Patel et al. 2008b). Depending on cell type, the number of caveolae on the membrane surface is different. Caveolae can increase the surface area of the plasma membrane up to 50% in adipocytes (Thorn et al. 2003, Thomas and Smart 2008).

A typical caveola is a 50-100 nm invagination in the plasma membrane. They are usually flask-shaped, but the morphology is dependent on the physiological status of the cells. The caveolae can also form tubular structures and vesicles, and one should regard them as being dynamic with several morphologies and containing different components (Thomas and Smart 2008) (for electron micrographs of caveolae see Fig. 1a and (Thorn et al. 2003, Parton and Simons 2007, Richter et al. 2008)).

Compared to the bulk membrane, which is mainly composed of glycerophospholipids with unsaturated acyl chains, the membrane rafts (i.e. the lipid rafts and caveolae) are enriched in cholesterol and sphingolipids containing saturated chains (de Laurentiis et al. 2007, Thomas and Smart 2008). Membrane rafts contain cholesterol, sphingomyelin, glycosphingolipid, phosphatidylinositol 4,5-biphosphate, the ganglioside GM1 and flottilin-1 and -2 (de Laurentiis et al. 2007) and ceramides (Anderson 1998). In addition, lipid rafts contain the ganglioside GM3, while caveolae contain the caveolin proteins, Cav1, Cav2 and Cav3 (de Laurentiis et al. 2007) and the cavin protein family (Thomas and Smart 2008, Bastiani et al. 2009). Many signaling proteins and receptors are also located to the membrane rafts, for further reading see section 5 and e.g. (Razani et al. 2002b, de Laurentiis et al. 2007).

In this thesis, the effect of caveolin on cellular processes and animal metabolism is discussed. However, also the lipid composition of the caveolae can affect signaling. For instance, cholesterol has been shown to affect caveolae. Upon cholesterol depletion, the caveolae can no longer keep their shape and appear flat (Thomas and Smart. 2008). The amount of caveolae in a cell and caveolin gene expression are linked to cholesterol concentration, wherein cholesterol depletion is able to decrease caveolin mRNA, and an increase in the level of free cholesterol is able to upregulate caveolin mRNA and the number of caveolae. Additionally, decreasing caveolin concentration lowers the rate of cholesterol efflux from the cells, thus suggesting a role for caveolin in cholesterol homeostasis. See further information in (Fielding and Fielding. 2000, Liu et al. 2002, Razani et al. 2002b, Frank et al. 2006, de Laurentiis et al. 2007, Thomas and Smart 2008). There are more lipids localized in the caveolae. However, these will not be discussed in this thesis. For further reading, see e.g. (Wang et al. 2002, Ortegren et al. 2004, Eyster. 2007, Prinetti et al. 2009, Sonnino and Prinetti 2009).

2.1 The caveolin proteins
Caveolin (Cav) proteins can be found in caveolae. Caveolin was first described as a substrate for phosphorylation in chick fibroblasts transformed with v-src (Rous sarcoma viral oncogene). In addition, caveolin was identified as VIP-21, a component of the trans-Golgi network. However, in 1992 Rothberg et al. linked caveolin to caveolae (Rothberg et al. 1992, Couet et al. 2001).

Three Cav proteins have been identified, Cav1, Cav2, and Cav3, with protein sizes of about 18 to 22 kDa. Both Cav1 and Cav3 can form invaginated caveolae, but Cav2 is not required for the formation. In cells that normally do not express Cav, Cav1 overexpression or Cav3 overexpression can induce caveolae formation (de Laurentiis et al. 2007, Thomas and Smart.
The formation of caveolae is thought to be dependent on caveolin oligomerization, its binding to cholesterol and interaction with glycosphingolipids (Okamoto et al. 1998). Although the caveolin proteins are mostly regarded to be localized with caveolae there is also evidence that they can exist outside the caveolae and act as signaling mediators (Head and Insel 2007), and in certain cell types they have been shown to localize to secretory vesicles and mitochondria (Li et al. 2001). The caveolin proteins residing in caveolae are generally thought to be distributed over the entire caveolae. However, Thorn et al. found that the caveolin localized to the neck of the caveolae in adipocytes (Thorn et al. 2003).

Williams et al. generated a phylogenetic tree of the caveolins in different species. In mammals, Cav1 has been confirmed to exist in cow, dog, mouse, rat, chicken, and human; Cav2 in dog, mouse, rat, and human; and Cav3 in mouse, rat, and human. Caveolins are also found in C. Elegans, Xenopus, and Fugu (Williams and Lisanti 2004). In Fig. 2, a phylogram of the caveolin and cavin proteins (see section 2.2) is shown.

Fig. 2. Phylogram of the caveolin and cavin proteins. The nucleotide sequences were taken from the nucleotide database in NCBI (National Center for Biotechnology Information), Cav1: NM_007616.3, Cav2: NM_016900.3, Cav3: NM_007617.2, cavin-1: NM_008986.2, cavin-2: NM_138741.1, cavin-3: NM_028444, and cavin-4: NM_026509.3. The phylogram was generated in the program ClustalW2.

The caveolins are hairpin-like structures with a hydrophobic membrane-spanning domain of 33 amino acids (although the caveolins are not really membrane-spanning, they are integral membrane proteins). The hydrophilic N- and C-terminus are both on the cytosolic side (de Laurentiis et al. 2007, Thomas and Smart 2008). The N-terminal part contains the caveolin scaffolding domain (CSD, 81-101 aa) which can interact with other proteins such as adenylyl cyclase, G_α, G_βγ, PKA, Src, PI3K, eNOS, PKC and Erk. The proteins are thought to bind in an inactive state, and activation leads to a conformational change that releases and activates the signaling proteins (Thomas and Smart 2008, Patel et al. 2008a). The N-terminal domain is also responsible for the interaction with other caveolin molecules to form oligomers (14-16 monomers) (Thomas and Smart 2008, Mercier et al. 2009). Additionally, the membrane-spanning domain has been implicated in the hetero-oligomerization of Cav1 with Cav2 (Das et al. 1999, Hnasko and Lisanti 2003). The C-terminal is thought to aid in the anchoring of the protein to the
plasma membrane (Thomas and Smart 2008). All three proteins contain the “caveolin signature motif” within the hydrophilic N-terminal domain (the “FEDVIAEP” stretch) (Razani et al. 2002b, Williams and Lisanti 2004).

The Cav1 protein has been investigated further. Both the N- and C-terminals contain so-called membrane attachment domains (MAD), called N-MAD (residues 82-101, this is the CSD), and C-MAD (residues 135-150) (see Fig. 3). The N-MAD domain directs Cav1 to caveolae, whereas C-MAD directs Cav1 to the trans-Golgi network. In the C-terminal domain, three cysteine residues (aa 133, 143 and 156) can be palmitoylated. This stabilizes the Cav1 structure at the membrane. The Cav1 protein also contains an oligomerization domain (residues 61-101) involved in the homo-oligomerization (Hnasko and Lisanti 2003, Williams and Lisanti 2004).

The caveolin proteins can be phosphorylated on Ser/Thr sites by e.g. PKCα and on Tyr sites by Src (de Laurentiis et al. 2007). See below for more information about specific phosphorylation for each Cav isoform.

![Fig. 3. Schematic view of Cav1. See text for explanation.](image)

**Caveolin-1**

*Cav-1* is located on chromosome 7q31.1 in human and on chromosome 6 in mouse. *Cav-1* has three exons with the sizes 30, 165 and 342 base pairs. The Cav1 protein has two isoforms, Cav1α and Cav1β. Cav1α is 178 amino acids long and Cav1β only 147 amino acids, due to a shorter N-terminus (Hnasko and Lisanti 2003, Williams and Lisanti 2004).

The chromosomal region where *cav-1* (and *cav-2*) is located is a region that is frequently lost in malignant tumors. This region is believed to contain a tumor suppressor gene, and *cav-1* (and *cav-2*) was thought to be the possible suppressor gene (Couet et al. 2001). Cav1 has now been shown to be a tumor suppressor; however, in some cancers it has also been shown to act as a tumor promoter (Razani et al. 2001, Williams and Lisanti 2005, Goetz et al. 2008, Mercier et al. 2009).
Cav1 is expressed in most cell types, but is highly enriched in adipocytes, smooth muscle cells, epithelial cells, endothelial cells, fibroblasts, and pneumocytes (Williams and Lisanti 2004, de Laurentiis et al. 2007, Thomas and Smart 2008.). Cav1 can localize to caveolae, the Golgi apparatus, trans-Golgi-derived transport vesicles (see section 2.3), and to secretory vesicles and mitochondria. The first 31 amino acids seem to be important in targeting Cav1 isoforms to different subcellular compartments. Depending on cell type, Cav1 may have a soluble cytoplasmic form and secreted form (Li et al. 2001, Williams and Lisanti 2004).

Cav1 can hetero-oligomerize with Cav2, and Cav1 is required for the proper localization of Cav2 (Williams and Lisanti 2004, Thomas and Smart 2008).

Regulation of the expression of Cav1 has been investigated (Quest et al. 2008). The expression of Cav1 has been shown to be under direct control of FOXO transcription factors (van den Heuvel et al. 2005); chronic β-adrenergic receptor stimulation in mice down-regulates Cav1 (and Cav3) (Oka et al. 1997); activation of PKA/Ras/Erk1/2 down-regulates Cav1 promoter activity (Engelman et al. 1999); (but) the PPARγ-agonist rosiglitazone can up-regulate Cav1 (by an EGFR, Src, MEK/Erk and p38 pathway) (Llaverias et al. 2004, Tencer et al. 2008); and nerve growth factor can inhibit glucose-induced down-regulation of Cav1 expression (Tan et al. 2003). Furthermore, age seems to affect Cav1 expression, with Cav1 being upregulated in senescent cells (Park et al. 2000, Volonte et al. 2002, Park et al. 2005), and an up-regulation of Cav1 in human adipose tissue is seen in obesity and in obesity-associated type 2 diabetes (Catalan et al. 2008). In addition, an effect of acclimation temperature has been observed in mouse brown adipose tissue, where the Cav1 protein levels were decreased with increasing acclimation temperature (paper I).

Adipocytes are highly enriched in Cav1 (and Cav2) and in their number of caveolae. In 3T3-L1 cells, differentiation into adipocytes increases both the expression of Cav1 (and Cav2, about 20-fold) and the number of caveolae (9-fold). Together with the fact that Cav can bind fatty acids, it is suggested that Cav1 and caveolae act together with other proteins in the uptake and transport of fatty acids into lipid droplets (Hnasko and Lisanti 2003, Ost et al. 2005) (see section 4). Cav1 and caveolae have also been implicated in insulin-stimulated GLUT (glucose transporter) translocation and thereby the glucose uptake into adipocytes (Scherer et al. 1994, Kandror et al. 1995, Karlsson et al. 2002).

Cav1 can be phosphorylated by many proteins, e.g. by src tyrosine kinases (Li et al. 1996), insulin (on Tyr14) (Mastick et al. 1995, Kimura et al. 2002), EGF (pathway via EGFR, Src, Ca\(^2+\), but not PI3K), and angiotensin II (pathway via EGFR, Src, Ca\(^2+\), and Ly-responsive PI3K) (Yin et al. 2008).
Phosphorylated Cav1 is a substrate for protein tyrosine phosphatase 1B (Lee et al. 2006).

**Caveolin-2**

Like cav-1, cav-2 is also located on chromosome 7q31.1 in human and on chromosome 6 in mouse. Cav2 was identified by Scherer et al. in 1996 and it has been shown to be co-expressed with Cav1. Cav-2 has three exons with the sizes 150, 188 and 151 base pairs. The Cav2 protein has three isoforms, Cav2α, Cav2β and Cav2γ. The Cav2α is the full-length protein and is 162 amino acids long. The two other isoforms have been identified, but are not characterized as of yet (Scherer et al. 1996, Hnasko and Lisanti 2003, Williams and Lisanti 2004).

Cav2 colocalizes with Cav1 and is found in the same cells as Cav1. Cav1 is required for proper location of Cav2 (which is otherwise retained in the Golgi where it is degraded). Cav2 cannot form caveolae by itself (Williams and Lisanti 2004, Thomas and Smart 2008).

Cav2 can be phosphorylated on Tyr19 and Tyr27 by Src and by stimulation with EGF. The different phosphorylations can affect Cav2 in both a spatial and temporal manner (Lee et al. 2002, Wang et al. 2004).

The expression of Cav2 has been linked to breast cancer (Savage et al. 2008).

**Caveolin-3**

Cav-3 is located on chromosome 3p25 in human and on chromosome 6 in mouse. Cav3 was identified by Tang et al. in 1996. Cav-3 has two exons with the sizes 114 and 342 base pairs, respectively, and the protein has only one isoform of 151 amino acids. Cav3 and Cav1 are approximately 65% identical and approximately 85% similar based on protein sequence homology (Tang et al. 1996, Hnasko and Lisanti 2003, Williams and Lisanti 2004).

Compared to Cav1 and Cav2, Cav3 is muscle-specific and can be found in skeletal, cardiac and smooth muscle cells. Cav3 is able to form caveolae, and does not require Cav1 for transport or to form oligomers (de Laurentiis et al. 2007, Thomas and Smart 2008).

Cav3 (and Cav1) expression can be down-regulated by chronic β-AR stimulation in mice (Oka et al. 1997).

Mutations in Cav3 have been found, which are associated with human muscular disease (Dowling et al. 2008).

2.2 The cavin proteins

Cavin was discovered in 2001 on the cytosolic face of caveolae. It is also called “polymerase I and transcript release factor” (PTRF) or “binding factor
of type-1 collagen promoter”. It colocalizes with Cav1 in human adipocytes. Cavin is expressed in various tissues, with a high abundance in adipocytes, lung, heart, and colon (Vinten et al. 2001, Pilch et al. 2007, Thomas and Smart 2008). Cavin has been implicated in lipid metabolism and colocalizes with hormone-sensitive lipase (Aboulaich et al. 2006). More recent studies have shown that cavin plays a critical role in caveolae formation and organization, and knock-down of cavin decreases the expression of Cav1. In 2008, Liu et al. generated a cavin-ablated mouse. These mice were viable but had no morphologically detectable caveolae in the tissues examined. Depending on the tissue, the mRNA levels of the caveolins were either normal or increased; however, the protein levels of the caveolins were reduced. The mice were of normal weight but had characteristics for a lipodystrophic phenotype (e.g. reduced adipose tissue mass, higher circulating triglyceride levels, glucose intolerance and hyperinsulinemia) (Hill et al. 2008, Liu and Pilch 2008, Liu et al. 2008).

Cavin belongs to the mammalian PTRF/SDR (SDR, serum deprivation response) family of proteins. Bastiani et al. found that the cavin proteins are coexpressed and can form a complex now called the Cavin complex. The four proteins are proposed to be named cavin-1 (the first identified cavin, also called PTRF), cavin-2 (/SDR), cavin-3 (/SRBC, SDR-related gene product that binds to C kinase) and cavin-4 (/MURC, muscle-restricted coiled-coil protein) (see Fig. 2 for phylogram). Compared to the other cavin proteins, cavin-4 is muscle-specific (Bastiani et al. 2009). The cavin proteins have a molecular weight between 31-47 kDa, and common structural motifs are leucine zipper(s), PEST domains (domain rich in proline (P), glutamic acid (E), serine (S) and threonine (T)) and putative phosphatidylserine-binding sites (Hayer et al. 2009). Cavin-1 and cavin-2 seem to play a role in the curvature of the caveolae membrane, while cavin-3 seem to play a role for the budding of caveolae (Nabi 2009).

In the years since cavin was discovered, more and more studies have been performed focused on the cavin proteins. With the generation of cavin-1-ablated mice and the relevance to caveolae formation, even more studies will undoubtedly yield further insights into the role of caveolae for cell signaling.

2.3 The formation and internalization of caveolae

The formation of caveolae is reviewed in e.g. (Parton et al. 2006, Parton and Simons 2007). The synthesis of caveolin occurs in the rough endoplasmatic reticulum (ER). Cav is synthesized as an integral membrane protein in a signal recognition particle-dependent manner (although cytosolic and secreted caveolins also seem to exist, see section 2.1). In most cells, caveolin seems to travel along the secretory pathway.
Already in the ER, it seems that the caveolins can oligomerize. From the ER, caveolin is transported to the Golgi complex. At this stage, caveolins are not associated with detergent-resistant membranes, and it seems Cav has not acquired its plasma membrane caveole characteristics. In the late Golgi compartment Cav1 is palmitoylated.

The transport from the Golgi is enhanced by addition of cholesterol and decreased by glycosphingolipid depletion. In the trafficking of caveolins from the post-Golgi, other components of caveolae are also assembled. Furthermore, the SNARE protein syntaxin-6 plays a role in the delivery of Cav1, GPI-anchored proteins, and the ganglioside GM1 to the plasma membrane. However, whether the caveola is already formed in the Golgi or first at the plasma membrane is still under debate. When the caveola is formed, it is very stable (Parton et al. 2006, Parton and Simons 2007). In a recent study by Hayer et al., the assembly of caveolae, especially with regard to Cav1, Cav2 and cavin-1, was investigated biochemically and by live-cell imaging. Newly assembled caveolin scaffolds were transported to the plasma membrane in vesicular carriers, and after arrival, cavin-1 was recruited to the caveolar domain (Hayer et al. 2009).

Endocytosis from the cell membrane can occur via clathrin-dependent mechanisms and clathrin-independent mechanisms, such as the CLIC/GEEC (CLIC: clathrin- and dynamin-independent carriers, GEEC: glycosyl phosphatidylinositol-anchored protein-enriched early endosomal compartments) endocytic pathway, arf6-dependent endocytosis, flotillin-dependent endocytosis, macropinocytosis, circular dorsal ruffles, phagocytosis and transendocytosis (reviewed in (Doherty and McMahon 2009)). Caveolae can also be endocytosed. For example cholera toxin, SV40 and GPI-linked proteins have been implicated to be endocytosed via caveolae (and lipid rafts). Endocytosed caveolae can fuse with early endosomes and the caveosome (is comparable to endosomes but without endosomal, ER or Golgi markers, and is rich in caveolin. For further reading, see (Pelkmans et al. 2001)). Sorting of the caveolae seems to be under the influence of Rab5. In addition, dynamin, Src kinases, PKC and actin seem to be involved in this endocytosis (Pelkmans and Helenius 2002, Parton and Richards 2003, Pelkmans et al. 2004, Parton et al. 2006, Parton and Simons 2007, Doherty and McMahon 2009), as well as cavin-3 (Nabi 2009). Certain receptors have been shown to internalize via caveolae (see section 5).

2.4 Methods to study caveolae

During the years since caveolae were discovered, many methods have been developed to be able to examine caveolin/caveolae and localization of recep-
tors and signaling mediators to these microdomains and their effect on signaling. Here I will briefly discuss methods involving cholesterol-disturbing agents, subcellular fractionation, colocalization, and the use of siRNA and overexpression. Cav-ablated mice have also been generated; however, these will be discussed in section 2.5.

Most of the studies on the interaction between receptors/mediators with caveolin or caveolae have been performed based on cholesterol disruptors (such as methyl-β-cyclodextrin (mβCD), filipin, and nystatin) and cellular fractionations. Both these methods have the problem of not only affecting caveolae but also lipid rafts and other components in the cells.

mβCD is widely used for removal of cholesterol from caveolae and lipid rafts. By removing cholesterol from the caveolae, the caveolae structure is disrupted. However, mβCD can remove cholesterol also from the bulk membrane, and it can alter the distribution of cholesterol between plasma and intracellular membranes. Additionally, phospholipids may be extracted by cyclodextrins (Zidovetzki and Levitan 2007, Patel et al. 2008a, Mahammad and Parmryd 2008). When performing experiments with mβCD, one should at least include control experiments by adding cholesterol-loaded mβCD.

Other cholesterol-disturbing agents are filipin and the statins (e.g. nystatin). Filipin does not remove cholesterol from the membrane but makes aggregates (Roepstorff et al. 2002), while statins deplete membrane cholesterol and reduces the amount of caveolae (Patel et al. 2008a). Cardiomyocytes have been shown to dislike filipin and nystatin (displayed gross cardiomyocyte toxicity before any changes in cellular cholesterol or cAMP could be detected); while no toxicity could be seen with mβCD (Rybin et al. 2000). However, whether cells are stable when using these agents or not seems to be a methodology- and/or a cell type-issue.

The first subcellular fractionations used detergents, such as Triton X-100 (e.g., to separate caveolae/lipid rafts from the bulk membrane (see (Sargiacomo et al. 1993, Chang et al. 1994)). However, the detergents are able to alter the molecular composition of the membrane rafts, and non-detergent alternatives have been developed; e.g. one method based on Percoll gradient/sonication/optiprep (see (Smart et al. 1995)); one based on carbonate buffer (pH 11)/sonication/sucrose gradient (see (Song et al. 1996)); and one based on cationic colloidal silica particles (see (Schnitzer et al. 1995)). Compared to the two first methods, this last method seems only to fractionate caveolae and not lipid rafts (Thomas and Smart 2008). Yet another method based on immuno-affinity isolation of caveolae has been developed which also separates the caveolae from the lipid rafts (Oh and Schnitzer 1999). Rat adipocyte caveolae have also been isolated with a similar method based on fractionation by sonication and then immunoabsorbption with antibodies against Cav1 (Souto et al. 2003). Thus, these would seem better if
one only wants to investigate caveolae and not caveolae/lipid rafts. When utilizing such methods as the above, protein markers for different compartments in the cell should be used, since certain methods can also fractionate proteins from mitochondrial and internal membrane origin (Mellgren 2008).

To ensure colocalization of receptors and mediators with caveolins, one should use methods such as co-immunoprecipitation and microscopy (preferably electron microscopy) (to ensure that only caveolae are investigated and not lipid rafts). However, the use of such methods has to ensure that antibodies and other labeling tools are specific against the proteins/lipids of interest. Additionally, since caveolins can be localized outside caveolae this poses a problem for the colocalization to caveolae. In the future, maybe the cavins would be better suited for these kinds of studies. Furthermore, if the desire is to investigate lipid rafts, microscopy is of no use since lipid rafts cannot be detected as yet.

The use of siRNA against different caveolins or other proteins can yield a better understanding of the specific role of a protein in a cell type. However, this requires transfection of cells and some cell systems are not easily transfected (e.g. primary brown adipocytes). In addition, the use of protein overexpression can give insight into the role of a specific protein. One caveat with this method is though that the overexpression may lead to non-physiological interactions. It is also important to investigate how the morphology of the caveolae is affected upon transfection. Patel et al. encourage scientists to use electron microscopy to ensure the effect of siRNA or overexpression (Patel et al. 2008a).

In recent years, the generation of different cav-ablated mice has been an important tool to investigate the effect of caveolin on cell signaling and animal physiology. This will be discussed in the next section.

2.5 Caveolin-ablated mouse models
Cav1-, Cav2-, Cav3-, and Cav1/3-ablated mouse models have been generated, and in this section, the findings surrounding these mice will be discussed. All of these mice are viable, and both Cav1- and Cav3-ablated mice show lack of caveolae in accordance with their expression patterns (i.e. Cav1-ablated mice lack caveolae in all tissues except heart and skeletal muscle, and Cav3-ablated mice lack caveolae only in heart and skeletal muscle). In the Cav1/3-ablated mouse, no caveolae can be found (Patel et al. 2008a).

Compared to some of the methods discussed in section 2.3, these mouse models are specific for the caveolin proteins. However, it should be noted that none of the mouse models are conditional-ablated mice, and as such the
observed phenotypes could be due to secondary effects (see further (Insel and Patel 2007)).

2.5.1 Caveolin-1-ablated mice

Four different Cav1-ablated mouse models have been generated. As seen in Fig. 4, one was made by targeted disruption of exon 3 (Drab et al. 2001), one by disruption of exon 2 (Cao et al. 2003) and two by the disruption of both exon 1 and 2 (Razani et al. 2001, Zhao et al. 2002). Drab et al. found that the ablated mice had vascular dysfunction and pulmonary defects (Drab et al. 2001); Razani et al. found that the Cav1-ablated mice had hyperproliferative and vascular abnormalities (Razani et al. 2001); Zhao et al. found that the mice had dilated cardiomyopathy and pulmonary hypertension (Zhao et al. 2002); and Cao et al. found that the Cav1-ablated mice had impaired renal calcium reabsorption which led to hypercalciuria (Cao et al. 2003).

![Cav-1 Exon Diagram](image)

Fig. 4. Generation of Cav1-ablated mice. Modified from (Le Lay and Kurzchalia 2005).

Many studies on these Cav1-ablated mice have been performed, especially on the mouse from Razani et al. since these are commercially available. To discuss all studies on the Cav1-ablated mice is beyond the scope of this thesis, and I will only briefly mention the phenotypes seen. As I discuss metabolism/brown adipose tissue and signaling in section 4 and 5, respectively, studies regarding these topics will not be discussed here. For further reading about Cav1-ablated mice, see e.g. (Hnasko and Lisanti 2003, Le Lay and Kurzchalia 2005, Patel et al. 2008a, Mercier et al. 2009).

Although the Cav1-ablated mice are viable, the mice generated by Razani et al. show dramatic reductions in life span (~50 % reduction). This could be due to secondary effects (Park et al. 2003, Le Lay and Kurzchalia 2005). As
will be discussed in section 4.2, Cav1-ablated mice are lean and resistant to diet-induced obesity (Razani et al. 2002). Cav1-ablated mice also show a loss of Cav2 protein (down to 10% of wild-type levels). This is not due to transcription, but dependent on trafficking of Cav2 by Cav1 (Hnasko and Lisanti 2003, Le Lay and Kurzchalia 2005).

Since Cav1 is expressed in many tissues, the effects of ablation are widespread. In the cardiac tissue there is a hyperactivation of Erk1/2 (leading to cardiac hypertrophy), abnormalities in the Src signaling and enhanced metalloproteinase-2-activity; in the vascular system there is increased eNOS (endothelial nitric oxide synthase) activity, altered VEGF signaling, increased levels of cyclin D1 and Erk1/2 and altered tight junctions; in the pulmonary system there is hyperphosphorylation of STAT3, activation of cyclin D1 and D3, the alveolar wall is thickened and there is a pulmonary hypertension; in the urogenital tract there is impaired renal calcium absorption, enlarged seminal vesicles and smooth muscle abnormalities leading to bladder hypertrophy; in skeletal muscle tissue there are muscle abnormalities (i.e. tubular aggregation); in neuronal tissue there is increased cerebral infarct volume and apoptosis, and motor and behavioral defects; in the endocrine-metabolic system there is altered lipid homeostasis, altered perilipin phosphorylation, accelerated mammary gland development (due to hyperactivity in prolactin signaling), exercise tolerance and decreased glucose uptake due to insulin resistance and altered glucose transporter localization. The Cav1-ablated mice also have high sensitivity to carcinogens and they have increased tumor permeability and growth (Hnasko and Lisanti 2003, Le Lay and Kurzchalia 2005, Patel et al. 2008a, Mercier et al. 2009).

2.5.2 Caveolin-2-ablated mice

Razani et al. generated a Cav2-ablated mouse (Razani et al. 2002a). As seen in Fig. 5, both exon 1 and 2 were disrupted (resulting in loss of all three Cav2 isoforms). These mice were found to have severe pulmonary dysfunction although the caveolae were not disrupted. Similarly to Cav1-ablated mice, the Cav2-ablated mice have hyperphosphorylation of STAT3, activation of cyclin D1 and D3, and thickened alveolar walls in the pulmonary system. Additionally, they are exercise-intolerant and have muscle abnormalities (due to tubular aggregation). Thus, the lung phenotype seen in the Cav1-ablated mice is most probably due to the decreased expression of Cav2 (Hnasko and Lisanti 2003, Le Lay and Kurzchalia 2005, Patel et al. 2008a, Mercier et al. 2009).
2.5.3 Caveolin-3-ablated mice

Two different Cav3-ablated mouse models have been generated. As seen in Fig. 6, both were generated by disruption of exon 2. Hagiwara et al. found that the ablated mice displayed muscle degeneration (Hagiwara et al. 2000), and Galbiati et al. found that the ablated mice had changes in the microdomain distribution of dystrophin-glycoprotein complexes and abnormalities in the T-tubule (Galbiati et al. 2001). In addition, the Cav3-ablated mice show hyperactivation of Erk1/2 in cardiac tissue (as did Cav1-ablated mice) according to Patel et al. who also observed a loss of caveolae-associated protective molecules (Patel et al. 2008a). In the endocrine-metabolic system, there is an increase in serum lipids and insulin resistance and altered glucose transporter localization (Hnasko and Lisaniti 2003, Le Lay and Kurzchalia 2005, Patel et al. 2008a, Mercier et al. 2009).

2.5.4 Caveolin-1/3-ablated mice

The Cav1/3-ablated mice were generated by interbreeding Cav1-ablated mice and Cav3-ablated mice (both on C57Bl/6 background). The double-ablated mice lacked both muscle and non-muscle caveolae. The ablated mice have lung, fat and skeletal defects to the same extent as their single-ablated genotypes; however, they also developed a more severe cardiomyopathic phenotype (Park et al. 2002, Le Lay and Kurzchalia 2005).
3. Brown adipose tissue

Compared to other species, mammals possess the specific organ brown adipose tissue. Brown adipose tissue is able to produce heat via the uncoupling protein 1 (UCP1, or thermogenin) resulting in so-called nonshivering thermogenesis. The function of this process is to survive cold temperatures, such as those during the night or winter, the infant’s exposure to cold after birth, but also for survival on diets low in essential nutrients (Cannon and Nedergaard 2004). The most studied animals concerning brown adipose tissue are rodents, such as mice and rats but also hibernating animals (e.g. ground squirrels and golden hamsters). In mice, brown adipose tissue exists in different locations, e.g. the interscapular, cervical, and axillary depots that are used for primary brown adipocyte cultures, but also the periaortic, perirenal and intercostal depots. For more detail, see (Cannon and Nedergaard 2004, Cinti 1999).

For many years, the general thought was that in humans only infants had brown adipose tissue (to survive cold after birth), and that it then gradually disappeared. However, in recent years, a new understanding has developed. Positron emission tomography (PET) is commonly used to trace tumor metastases. However, the images obtained were often confused by signals that were not due to metastases, and most probably they were active brown adipose tissue. Compared to rodents they were located differently, and the main depots were found in the supraclavicular and the neck regions (and also along the spinal cord) (Nedergaard et al. 2007). In the years since, studies using PET to directly assess the amount of brown adipose tissue were performed. It was found that brown adipose tissue was functional (with expression of UCP1), that it was activated upon cold exposure and that it possibly correlates negatively with both obesity and age (van Marken Lichtenbelt et al. 2009, Virtanen et al. 2009, Zingaretti et al. 2009, Saito et al. 2009) (see also comments in the same issue of NEJM and also replies some months later). Thus, brown adipose tissue could possibly be a tool in treating obesity and metabolic disorders.

In the next section, I will discuss some properties of brown adipose tissue and then in section 3.2 discuss signaling to lipolysis and nonshivering thermogenesis. For further reading about brown adipose tissue, see review by (Cannon and Nedergaard 2004).
3.1 Properties of brown adipose tissue

For many years, brown adipose tissue (BAT) has been regarded to be most closely related to white adipose tissue (WAT). However, there are some major differences, e.g. that WAT is an energy-storing tissue while BAT is an energy-consuming tissue. Additionally, BAT expresses the unique protein UCP1, has a high number of mitochondria and has multilocular lipid droplets as compared to WAT (no UCP1, fewer mitochondria, and only one large lipid droplet) (Cinti 1999, Tiraby and Langin 2003).

In recent years, it has become more evident that brown and white adipocytes are not so easily distinguished, with cells derived from white adipose depots displaying more brown adipocyte-like features (i.e. being able to express UCP1 upon norepinephrine-stimulation). These cells are proposed to be called “brite” adipocytes (Lehr et al. 2009, Petrovic et al. 2009). Additionally, there have been suggestions that both brown and white adipocytes are able to transdifferentiate into each other (Tiraby and Langin 2003, Cinti 2009).

To add to this complexity, recent research has shown that brown adipocytes are more closely related to myocytes (than to white adipocytes), with the brown adipocytes expressing myogenic markers and muscle-specific microRNAs (myomirs). Thus, brown adipocytes and myocytes derive from a common cell lineage (Atit et al. 2006, Timmons et al. 2007, Walden et al. 2009). The decision to either become a brown adipocyte or a myocyte seem to be regulated by PRDM16 (Seale et al. 2007, Seale et al. 2008) and BMP7 (Tseng et al. 2008).

The Wnts also seem to play a role in the development of brown (and white) adipocytes, with e.g. Wnt10b inhibiting the development of adipocytes (Longo et al. 2004, Christodoulides et al. 2009).


As for any other cell, the life span of brown adipocytes can be divided into proliferation, differentiation, and apoptosis. In the following paragraphs I will discuss the regulation of brown adipose tissue, where norepinephrine is one of the key regulators. Norepinephrine is released via the sympathetic nervous system (under regulation of the central nervous system). During cold acclimation, an increase in NE release can be seen (see (Cannon and Nedergaard 2004) for the activation of the sympathetic release of NE). NE can stimulate all the adrenergic receptors present in BAT, i.e. $\beta_1$-$\beta_3$, $\alpha_2$-, and $\alpha_1$-adrenergic receptors (see chapter 5.1-3 for more information) (Cannon and Nedergaard 2004).

In pre-adipocytes, NE stimulates cell proliferation through a $\beta_1$-adrenergic receptor/cAMP pathway (see also section 5.1) (Bronnikov et al.
1992). The transcription factor C/EBPα and the nuclear hormone receptor PPARγ2 are down-regulated by NE, and this is thought to be necessary for proliferation to proceed. In pre-adipocytes, UCP1 expression cannot be induced by NE, and thus, pre-adipocytes do not induce heat production via UCP1 (Cannon and Nedergaard 2004). Both the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) can induce cell proliferation via the extracellular-regulated protein kinase1/2 (Erk1/2) pathway (see paper II and section 5.5-6). LPA can also activate Erk1/2 (paper III) and it may play a role in proliferation control since it has been shown to downregulate PPARγ in both brown adipocytes (unpublished observations) and white adipocytes (Simon et al. 2005) (see section 5.4).

Also in differentiation of the brown adipocytes, norepinephrine (NE) plays a role. The differentiation process involves the possibility to increase UCP1 gene expression by NE and an increase in the amount of mitochondria; leading to an increase in the capacity for fatty acid catabolism for nonshivering thermogenesis (Cannon and Nedergaard 2004).

The differentiation is thought to occur through a cAMP-dependent pathway, either through direct effects on expression of certain genes by the cAMP/PKA/CREB (cyclic adenosine monophosphate/protein kinase A/cAMP response element binding protein) pathway, or indirect effects with NE increasing expression of certain transcription factors that can switch on differentiation, and it is known that NE increases the levels of both C/EBPβ (CCAAT enhancer binding protein β) and PGC-1 (PPARγ coactivator 1) (Cannon and Nedergaard 2004). In addition, p38 MAP kinase has been implicated as a mediator between cAMP/PKA and downstream targets (Cao et al. 2004).

UCP1 is the protein responsible for nonshivering thermogenesis. Upon differentiation, the mRNA levels of UCP1 are increased (if in the presence of NE, see also section 5.1-2). This gene expression is regulated by both a proximal promoter and a distal complex enhancer region. The proximal promoter contains a TATA-box, CCAAT elements, a cAMP response element (CRE) where CREB can bind, two C/E sites where C/EBP can bind, and also other sites (possibly an ETS TF site and a BRE region). The complex enhancer region has many response elements such as the CREs, the PPAR response elements (PPRE) for binding of peroxisome proliferator activated receptors (PPAR, both PPARα and PPARγ can bind), the retinoic acid response elements (RARE) with sites for RXR and RAR, and the thyroid responsive elements (TRE) where thyroid hormone can bind (T3 – triiodothyronine). The different response elements have been found to be able to affect UCP1 expression. Treatment with PPARγ agonist increases UCP1 gene expression. Retinoic acids can also increase UCP1 expression, but the increase is dependent on the cells being simultaneously differentiated. The thyroid hormone
receptor (TR) is in the non-ligand-bound state a repressor of UCP1 expression; however, upon ligand binding this repression is inhibited and thus, T₃ has a positive effect on UCP1 expression. In agreement with this, the TR-ablated mice express UCP1 upon NE stimulation, and the TR in itself is not necessary for UCP1 expression (Cannon and Nedergaard 2004, Golozoubova et al. 2004).

Regulated (or programmed) cell death (or apoptosis) of brown adipose tissue is inhibited by NE and activated by TNFα (tumor necrosis factor α). NE inhibits apoptosis by activation of β- and α₁-adrenergic receptors, and mice subjected to cold (leading to sympathetic stimulation) have decreased apoptosis (Lindquist and Rehnmark 1998, Cannon and Nedergaard 2004). TNFα is thought to elicit its pro-apoptotic effects via p38, and NE seems to be able to protect against the TNFα-induced apoptosis (Cannon and Nedergaard 2004).

Although the discussion above has focused on the brown adipocytes, brown adipose tissue not only contains brown mature adipocytes and brown pre-adipocytes; endothelial cells from capillaries and interstitial cells are also present (for further reading about BAT morphology see (Cinti 1999, Cinti 2009)). Upon demand for expansion of the tissue (proliferation and differentiation), not only the brown adipocytes will be affected as seen above, but also the amount of capillaries and nerve terminals will be increased (Cannon and Nedergaard 2004, Cinti 2009, Xue et al. 2009).

### 3.2 Lipolysis and nonshivering thermogenesis

Brown adipose tissue is able to produce heat via nonshivering thermogenesis. Both exposure to cold and recruiting diets activate brown adipose tissue and nonshivering thermogenesis. A normal mouse (or naked human) has its thermoneutral zone at about 30 °C. Upon acute exposure to cold (e.g. the transfer to 4 °C), the animal will try to defend its body temperature. Initially BAT has not been recruited and the animal will have to depend on shivering. However, during prolonged exposure to cold (several weeks), BAT is recruited and nonshivering thermogenesis will replace shivering (Cannon and Nedergaard 2004). Since most studies done on mice have been performed at room temperature (22 °C), these mice will have some recruited BAT and can survive in cold with a combination of shivering and nonshivering thermogenesis. Also the exposure to different food diets, such as high-fat diet and cafeteria diet, leads to a recruitment of BAT. This recruitment could have been an evolutionary “purpose” to allow animals to survive on diets low in essential nutrients (Cannon and Nedergaard 2004).
The activation of lipolysis and nonshivering thermogenesis by norepinephrine (NE) is dependent on β3-adrenergic receptors (β3-AR) (see Fig. 7), but also α1-adrenergic receptors can influence nonshivering thermogenesis (maximal influence reported is only 10% of the total response). Upon NE stimulation, β3-AR stimulates Gs-proteins, which in turn activates adenylate cyclase to produce cAMP. cAMP activates PKA which can phosphorylate both hormone sensitive lipase (HSL) and perilipin. The phosphorylation of perilipin leads to the dissociation of perilipin from the lipid droplet and the droplet is now accessible to both ATGL (adipocyte triglyceride lipase) and activated HSL. ATGL and HSL will break down the triglycerides into glycerol and free fatty acids (FFA) (Cannon and Nedergaard 2004, Zimmermann et al. 2004) (for further reading about ATGL and HSL see (Zimmermann et al. 2004, Pinent et al. 2008, Zimmermann et al. 2009)).

Fig. 7. Lipolysis in brown adipose tissue. For explanation, see text.
Some of the FFA will be bound by fatty acid binding protein (FABP, e.g. FABP4 (or aP2)). The fatty acids are the substrates for the respiratory chain and are transferred into the mitochondria and β-oxidized. In contrast to other tissues that do not express UCP1, brown adipose tissue can use fatty acids to induce nonshivering thermogenesis by the uncoupling effect of UCP1. The fatty acids can activate UCP1, while purine nucleotides, experimentally GDP (but also GTP, ADP and ATP), inhibit UCP1 function (Cannon and Nedergaard 2004). Glucose is also a good substrate for thermogenesis.

One would expect that both β3-AR-ablated mice and UCP1-ablated mice would have a difficult time surviving in prolonged cold due to impairment of their lipolysis and nonshivering thermogenesis capacity. However, this is not the case for either of these mice. Signaling by the β3-AR in the β3-AR-ablated mice is rescued by β1-AR and α1-AR signaling (Chernogubova et al. 2005) and the mice are able to survive in cold and induce nonshivering thermogenesis in response to NE to the same extent as wild-type mice (unpublished observations).

UCP1-ablated mice can survive weeks/months in cold if they have been successively exposed to colder temperatures (they cannot, however, maintain body temperature upon acute exposure to cold). The survival was not due to a recruitment of nonshivering thermogenesis, but due to the fact that the acclimation to decreasing temperatures allowed the mice to increase their muscle activity for shivering and to increase their physical endurance in general (Golozoubova et al. 2001). UCP1-ablated mice acclimated to thermoneutrality become obese on both normal diet and, when fed a high-fat diet, this weight gain was further augmented. Thus, these mice were not able to induce diet-induced thermogenesis (Feldmann et al. 2009).
4. Caveolin and metabolism in adipose tissue

Caveolin-1 (Cav1)-ablated mice are resistant to diet-induced obesity (Razani et al. 2002) and are unable to maintain body temperature when acutely exposed to cold and fasting (Cohen et al. 2005). Cav1-ablated mice also show impaired lipolysis in both white and brown adipocytes (Cohen et al. 2005, Cohen et al. 2004, paper I), and Cav1 has been suggested to play a role in lipid droplet formation (Ortegren et al. 2007, Le Lay et al. 2009). In this section, I will discuss these findings and compare them to those found in our group. Since lipolysis is adrenergically regulated (especially by $\beta_3$-adrenergic receptors), some of these findings will also be discussed in section 5.1; however, in section 5.1, it is more in the context of signaling than concerning the physiology of the mouse.

Caveolins can bind both cholesterol and fatty acids, and caveolins have been shown to be present on the surface of lipid droplets, and they seem to play a role in cellular lipid homeostasis. Caveolins can regulate the cholesterol content of the lipid droplets in adipocytes. Upon addition of cholesterol to 3T3-L1 cells, endocytosis of caveolae was seen (Le Lay and Kurzchalia 2005, Le Lay et al. 2006). Also, Cohen et al. found that Cav1 was involved in lipid droplet formation, since Cav1(-/-) mouse embryonic fibroblasts (MEFs) transfected with perilipin did not accumulate as much lipids as the perilipin-transfected wild-type MEFs (Cohen et al. 2004).

Fatty acid esterification is thought mainly to take place in the endoplasmatic reticulum (Le Lay et al. 2009). However, as discussed in Le Lay et al., Ost et al. found that triglycerides could be synthesized in a specific subclass of caveolae that also contained perilipin (Ost et al. 2005), and thus maybe adipocytes have one pathway to take care of exogenous fatty acids and another pathway to take care of endogenous fatty acids (from de novo lipogenesis or lipolysis of stored triglycerides). Since caveolae (and lipid rafts) are detergent-resistant, the thought of transport via caveolae is not far-fetched (Le Lay et al. 2009). Blouin et al. investigated the effect of Cav1-ablation on the lipid droplet proteome and lipidome. They found that there were alterations in both protein and lipid content, and possibly that Cav1 has a role in lipid droplet expandability (Blouin et al. 2009).
4.1 Cav1-ablated mice: resistance to diet-induced obesity

Caveolin-1 (Cav1)-ablated mice living at room temperature on a normal (chow) diet show the same growth curve as wild-type mice under the same conditions (Razani et al. 2002, paper I). However, as the mice got older (at about one year of age), Cav1-ablated mice displayed a leaner phenotype than the wild-type mice (these mice were not back-crossed though) (Razani et al. 2002). As seen in paper I, Cav1-ablated mice have lower fat content and higher lean content compared to wild-type mice. For Cav1-ablated mice to become leaner than wild-type mice, the energy balance between these genotypes must be different; i.e. the energy intake must be lower and/or the energy output must be higher in Cav1-ablated mice. No difference in food intake can be seen (Razani et al. 2002, paper I), except for that in the study by Razani et al., the female Cav1-ablated mice actually ate more than the female wild-type mice (Razani et al. 2002). No differences could be seen in oxygen consumption or on substrate used (respiratory quotient) (Razani et al. 2002, paper I) (despite the observation that the Cav1-ablated mice have abnormalities in the lungs and are exercise-intolerant (Drab et al. 2001, Razani et al. 2001)). Razani et al. could not see any differences in released heat or activity (Razani et al. 2002); however, in our study, the Cav1-ablated mice were less active/“agitated” in the start of the measurements (paper I). Thus, these results did not explain the lean phenotype (i.e. not higher metabolism/activity). No differences could either be seen in stool triglycerides (Razani et al. 2002), thus, there was not a decreased uptake of triglycerides. Thus, Cav1-ablated mice were not lean due to reduced food intake, increased metabolism/activity, or reduced triglyceride uptake.

To investigate whether Cav1-ablated mice had altered metabolite storage, serum metabolites were investigated (Razani et al. 2002). No differences could be seen in serum insulin, glucose, or cholesterol, neither in a fasted state nor postprandially. However, differences could be seen in both serum triglycerides (TG) and free fatty acids (FFA). In the fasted state, Cav1-ablated mice had increased serum TG, which was further augmented postprandially. In the fasted state, the levels of serum FFA were the same, but the reduction in FFA acids seen in the wild-type mice postprandially could not be seen in Cav1-ablated mice. In addition, both plasma leptin and adiponectin levels were lower in Cav1-ablated mice (Razani et al. 2002) (however, most probably the reduction in leptin levels correlates to the reduced adipose tissue mass).

In addition, lipoprotein distribution has been investigated in both chow-and high-fat diet (HFD)-fed wild-type and Cav1-ablated mice (Razani et al. 2002, Heimerl et al. 2008, Frank et al. 2008). Cav1-ablated mice are resistant
to diet-induced obesity and on a HFD, Cav1-ablated mice have less fat content compared to wild-type mice (Razani et al. 2002).

On a chow diet, Razani et al. found that the chylomicron/VLDL fraction in the Cav1-ablated mice had increases in both cholesterol and triglycerides levels after fasting and these were even further augmented postprandially (Razani et al. 2002). Heimerl et al. investigated the effect of both chow diet and HFD on fasting and postprandial plasma lipids and lipoproteins from Cav1-ablated and wild-type mice. The results were in agreement with the above study with regard to the effect seen on the chylomicron/VLDL fraction. However, Cav1-ablation also affected the HDL-cholesterol metabolism, and a different distribution of apo-AI/apoE could be seen (Heimerl et al. 2008). In addition, Frank et al. found that Cav1-ablated mice on chow diet showed a reduced VLDL-secretion from the liver, but also an increase in HDL, which was also enriched in cholesteryl esters in comparison to the wild-type mice. Cav1 was also implicated in the regulation of plasma LDL levels (Frank et al. 2008). Furthermore, Cav1-ablated mice had a delayed triglyceride clearance, even though the lipoprotein lipase activity was found to be the same (Razani et al. 2002).

If adipocytes cannot store lipids properly, this could lead to an increase of lipids in other organs. In HFD-fed Cav1-ablated and wild-type mice, no difference could be seen in liver tissue weight, thus the liver steatosis was the same for both Cav1-ablated and wild-type mice. However, the brown adipose tissue was enlarged in the ablated mice at 9-months of age (Razani et al. 2002), and thus, brown adipose tissue could be a regulator of lipid storage (see also section 4.2.2-3).

In perigonadal fat pads, Cav1-ablated mice show insulin resistance and on a HFD, Cav1-ablated mice developed postprandial hyperinsulinemia. In the perigonadal fat pads, protein expression of the insulin receptor was reduced (by 90%), and the protein levels of GLUT4 and PKB/Akt were increased. However, both the insulin-induced phosphorylation of PKB/Akt and the insulin-induced dephosphorylation of GSK were inhibited. Thus, since insulin-regulated lipogenesis is impaired, this could be a contributing factor to the lean phenotype seen in the Cav1-ablated mice (Cohen et al. 2003).

In conclusion, although differences in serum metabolites and impairment in both lipogenesis and lipolysis have been found, this still does not fully explain the lean phenotype observed in the Cav1-ablated mice and the resistance to diet-induced obesity – the Cav1-ablated mice are leaner even though the energy intake and the energy output seem to be the same (which is an impossibility).

Mice living at room temperature (22 °C) are under chronic thermal stress, and to defend their body temperature they have an increased metabolism and...
higher food intake (Feldmann et al. 2009, paper I). To overcome this problem when investigating diet effects, mice should be housed at thermoneutrality. Therefore, female Cav1-ablated and wild-type mice were acclimated to thermoneutrality (30 °C) and put on either a chow or a high-fat diet. Preliminary data show that, as described before (Razani et al. 2002), the Cav1-ablated mice are resistant to diet-induced obesity (Fig. 8A). No differences could be seen in energy intake between ablated and wild-type mice; both had increased energy intake on HFD (Fig 8B), and thus, Cav1-ablated mice had lower food efficiency (Fig. 8C)). The lean phenotype seen in Cav1-ablated mice on chow diet could possibly be explained by an increased amount of feces (i.e. if the triglyceride content is the same as seen by (Razani et al. 2002)), but on a HFD there was no difference in feces amount (Fig. 8D).

As expected (paper I), there was no difference in resting or mean metabolic rate (RMR or MMR) in mice fed chow diet (Fig. 9AB). However, Cav1-ablated mice on HFD had both lower RMR and MMR compared to the wild-
type mice (Fig. 9AB); thus even a lower metabolism than the wild-type mice. In response to norepinephrine, there was both a significant effect of genotype (as expected on chow diet (paper I)) and diet (Fig. 9CD). Thus, diet-induce thermogenesis seems to occur in both wild-type and Cav1-ablated mice.

However, these data do not explain why Cav1-ablated mice are resistant to diet-induced obesity. Cav1-ablated mice even had a lower metabolism on HFD. Thus, further studies are needed to understand how Cav1-ablated mice are able to not become obese on a high-fat diet.

Fig. 9. Cav1-ablated mice and wild-type mice acclimated to thermoneutrality on either chow or high-fat diet. A) Resting metabolic rate (RMR) calculated per lean body mass. B) Mean metabolic rate (MMR) calculated per lean body mass. CD) Anaesthetized mice (n = 4 - 6) were injected with norepinephrine (NE, 1 mg/kg s.c.). C. Oxygen consumption before and after norepinephrine injection. D. Increase in oxygen consumption by norepinephrine calculated per lean body mass.
4.2 Cav1-ablated mice: lipolysis and cold tolerance

4.2.1 Lipolysis in white adipocytes: fasting and β3-agonist stimulation

Two studies have investigated the role of Cav1 in the lipolysis pathway in white adipocytes (Cohen et al. 2004, Ahmad et al. 2009). Upon fasting for 48 h, wild-type mice were found to increase their levels of Cav1 and when re-fed, the levels returned to normal. In addition, fasting led to a rise in serum free fatty acid (FFA). However, in the Cav1-ablated mice, this rise could not be seen; and thus there seemed to be an impairment in lipolysis (Cohen et al. 2004).

Since lipolysis is activated through β3-adrenergic receptor (β3-AR) stimulation, glycerol and FFA levels in response to the agonist CL316, 243 (CL) were investigated. Without stimulation, no differences between Cav1-ablated and wild-type mice could be seen in glycerol levels in isolated adipocytes, in serum glycerol or serum FFA. However, upon CL-stimulation, only wild-type mice had a rise in these metabolites (Cohen et al. 2004). Thus, Cav1-ablated mice had also an impaired response to β3-adrenergic stimuli.

In perigonadal fat pads, there were no differences in β3-AR protein levels. However, Cav1-ablated mice had higher levels of the regulatory subunits of PKA (both RI and RIIα), but the PKA activity was higher. Upon CL-stimulation, Cav1-ablated mice had higher phosphorylation levels of CREB but lower levels of phosphorylated perilipin (see section 5.1) (Cohen et al. 2004). In agreement, Cav1 siRNA-treated 3T3-L1 cells reduced the levels of phosphorylated perilipin and hormone-sensitive lipase (HSL) (Ahmad et al. 2009).

Stimulation with CL can induce protein/lipid complex formation in both 3T3-L1 cells and perigonadal fat pads. Cohen et al. found that upon CL-stimulation, a complex between Cav1/perilipin/cPKA (c: catalytic) is formed in both 3T3-L1 cells and perigonadal fat pads. However, in the Cav1(−/−) fat pads, CL cannot induce this complex (Cohen et al. 2004). In agreement with this, Ahmad et al. found that knock-down of Cav1 in 3T3-L1 cells inhibited the CL-induced complex formation of cholesterol, 14-3-3, PP2A, Cav1, β3-adrenergic receptor, PKA-RII and HSL (Ahmad et al. 2009). Ahmad et al. also found that knock-down of Cav1 in 3T3-L1 cells reduced the CL-induced activation of phosphodiesterase 3B (PDE3B, hydrolyses cAMP to AMP). This was also seen in epididymal fat pads from Cav1-ablated mice (Ahmad et al. 2009).

In conclusion, in both white adipose tissue and the cell-line 3T3-L1, Cav1 is involved in the regulation of lipolysis, and Cav1-ablation leads to an impairment of lipolysis.
4.2.2 Effect of acute cold and fasting

Cav1-ablated mice were found to have an impairment in white adipocyte lipolysis. To investigate whether also brown adipocyte lipolysis was impaired, Cohen et al. investigated the effect of acute cold and/or fasting on Cav1-ablated and wild-type mice (Cohen et al. 2005).

Already under normal conditions, the Cav1-ablated mice had lower body temperature than the wild-type mice. In response to acute cold, there was no difference in body temperature between wild-type and Cav1-ablated mice (both had a drop in body temperature), but in response to fasting, the body temperature was decreased more in Cav1-ablated mice. When exposed to both acute cold and fasting (for 24 h (cold/fasting)), the body temperature was decreased for both wild-type and Cav1-ablated mice. However, Cav1-ablated mice could not maintain their body temperature and a marked drop could be seen (Cohen et al. 2005).

No differences could be seen in basal serum NEFA (non-esterified fatty acid) between wild-type and Cav1-ablated mice. However, upon 24 h fasting or cold/fasting, the Cav1-ablated mice were not able to raise their serum NEFA levels. There was, however, no difference in the serum triglycerides between wild-type mice or Cav1-ablated mice in either normal state or after cold/fasting (with levels reduced for both after cold/fasting) (Cohen et al. 2005).

In the brown adipose tissue, Cav1-ablated mice were not able to mobilize triglycerides after cold/fasting; the levels were not reduced as much as in the wild-type mice (Cohen et al. 2005). Thus, an impairment in brown adipocyte lipolysis could be seen. Investigating the pathway of lipolysis in brown adipose tissue in the mice exposed to cold/fasting, Cav1(-/-) brown adipose tissue had an impairment in phosphorylation of perilipin (not increased as in wild-type) and in HSL levels (is increased in Cav1(-/-), not in wild-type). No differences could be seen in the fatty-acid binding proteins CD36 and aP2 (Cohen et al. 2005). However, CD36 has been found to have different subcellular distribution. In agreement with Cohen et al., Ring et al. found that there was no difference in the expression of CD36 in wild-type MEFs (mouse embryonic fibroblasts) and Cav1(-/-) MEFs, but due to the difference in subcellular distribution, reduced fatty acid uptake in the Cav1(-/-) MEFs could be seen (Ring et al. 2006). Thus, although Cohen et al. did not see any differences on expression levels, there still could be differences in the subcellular distribution of the CD36 also in the brown adipose tissue.

The lipolysis of triglycerides to fatty acids takes place in the cytosol, but the fatty acids are oxidized in the mitochondria. Thus, the impairment seen in Cav1-ablated mice could be in the mitochondria. Cav1(-/-) brown adipose tissue mitochondria were larger, dilated and less electron dense than wild-type mitochondria. However, the integrity of the inner and outer mitochondrial membranes was not different. In addition, there was no difference in
UCP1, Hsp60 and prohibitin levels between the wild-type and Cav1-ablated mice (although UCP1 did increase in response to cold/fasting in both wild-type and Cav1-ablated mice) (Cohen et al. 2005). In agreement with this, we did not see any difference in the UCP1 protein levels between Cav1-ablated and wild-type mice (paper I). However, Cav1-ablated mice were not able to reduce levels of mitochondrial dicarboxylate carrier protein (mDIC) or increase phosphorylation of the catalytic subunit of AMPK (adenosine monophosphate kinase) in response to cold/fasting as did the wild-type mice (Cohen et al. 2005). To investigate whether the brown adipose tissue mitochondria were functional, mitochondria from Cav1-ablated and wild-type mice were isolated to determine thermogenic parameters (paper I). We found that the mitochondria from Cav1-ablated mice were able to respond to both carbohydrate (pyruvate) and lipid (palmitoyl CoA), and that the response was through UCP1 (was inhibited by GDP (guanosine diphosphate) and this inhibition could be overcome by FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazone, an artificial uncoupler)). Thus, even though certain proteins and structures are affected in the mitochondria, the UCP1 and the mitochondria are functional.

In conclusion, Cav1-ablated mice are not able to maintain body temperature in response to a combination of acute cold and fasting. An impairment in brown adipose tissue lipolysis could be seen and in some of the lipolytic signaling mediators.

4.2.3 Effect of prolonged cold

Since the Cav1-ablated mice have impaired lipolysis and could not maintain body temperature when exposed to cold and fasting, it was speculated that the Cav1-ablated mice would not be able to survive prolonged exposure to cold due to impaired nonshivering thermogenesis (Cohen et al. 2005). However, in response to acute cold, brown adipose tissue has not had a chance to become recruited and nonshivering thermogenesis does not occur (or only barely, if a little brown adipose tissue is present) – instead mice have to rely on shivering for this acute exposure to cold.

To investigate whether the Cav1-ablated mice would be able to survive prolonged time in cold by nonshivering thermogenesis or whether they could use a compensatory mechanism, wild-type and Cav1-ablated mice were acclimated to cold (2 weeks at 18 °C, then placed at 4 °C). As seen in paper I, the Cav1-ablated mice were able to survive, and this was through nonshivering thermogenesis. We could show that the Cav1-ablated mice have desensitized adrenergic receptors, but they are able to overcome this physiologically and thus are able to survive comfortably in cold. As discussed in the paper, this is similar to the desensitization seen in thyroid hormone receptor (TR)-ablated mice (Golozoubova et al. 2004). As shown in paper I, Cav1-ablation did not impair UCP1 protein expression and the UCP1 was functional. How-
ever, Cav1-ablated mice had a lower protein expression of β3-adrenergic receptors.

Cav1 has been shown to play a role in proper thyroid hormone synthesis, and Cav1-ablated mice have reduced levels of T₃ (Senou et al. 2009). T₃ has been shown to affect adrenergic receptor expression (Rubio et al. 1995) and thus, the decrease in β3-adrenergic receptor protein expression in brown adipose tissue could be due to the reduced levels of T₃.

In conclusion, in response to prolonged cold, brown adipose tissue is activated, and Cav1-ablated mice survive by nonshivering thermogenesis, despite having desensitized adrenergic receptors.
5. Caveolin and signaling

Many different receptor types exist in the plasma membrane of cells. In the following section, some of receptors relevant to brown adipose tissue signaling and function will be discussed, as well as the influence of caveolin-1 on their signaling. These receptors are either G-protein coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs). The GPCRs are seven-transmembrane receptors, with the N-terminus on the extracellular side and the C-terminus on the intracellular side (Hein and Kobilka 1997, Saunders and Limbird 1999, Garcia-Sainz et al. 1999, Dorsam and Gutkind 2007), and couple, as indicated by their name, to G-proteins. Discussed below are the adrenergic receptors (ARs), β-, α2- and α1-AR, and the lysophosphatidic acid receptors (LPA Rs). The RTKs have only one transmembrane domain and, upon ligand binding, they dimerize to become active. Discussed below are the epidermal growth factor receptors (EGFRs) and the platelet-derived growth factor receptors (PDGFRs). The insulin receptor and other signaling mediators relevant to brown adipose tissue signaling and function are also briefly discussed.

5.1 β-adrenergic receptors

There are three subtypes of the β-adrenergic receptors (β-AR), the β1, β2, and β3 (Bylund et al. 1994), with the β3-AR in mouse having two splice variants, β3a- and β3b-AR (Evans et al. 1999). The existence of a fourth β-AR has also been discussed (Kaumann 1997, Preitner et al. 1998). The β1-, β2-, and β3-AR are differentially expressed, with e.g. β1-AR being highly expressed in heart and brain, β2-AR expressed in skeletal muscle, lung, uterus, and prostate, and β3-AR highly expressed in adipose tissue (Frielle et al. 1987, Emorine et al. 1989, Strosberg 1997).

In brown adipose tissue, mRNA for all β-AR subtypes can be found. However, in cultured cells the expression of the β2-ARs are only detectable at low level after stimulation with NE and then only transiently (Bengtsson et al. 2000), and it is possible that the receptors are predominantly localized to the vascular system in the brown adipose tissue (Cannon and Nedergaard 2004). Of the two different β3-AR splice variants, it is mainly the β3a-AR mRNA that is expressed in brown adipose tissue (β3a-AR mRNA are about
9/10 of all β3-AR mRNA) (Evans et al. 1999). In mice acutely exposed to cold, the β3-AR mRNA is reduced transiently and returns to control levels after 24 h. In mice acclimated to prolonged cold, the β3-AR mRNA levels are not different from control levels. In contrast, the β1-AR mRNA in mice acutely exposed to cold is increased transiently and then reduced to control levels (Bengtsson et al. 1996, Bengtsson et al. 2000). In primary brown adipocyte cell cultures, the mRNA level of the β1-AR mRNA peaks during the proliferative phase and then decreases with time, while the β3-AR mRNA expression level is upregulated with time (peak when differentiation is initiated) (Bronnikov et al. 1999). Chronic treatment on primary cultures with NE also reduces the β3-AR mRNA level transiently, and the mRNA level returns back to control levels after about 15 h. In contrast, the β1-AR mRNA levels is increased upon chronic NE stimulation and remains at high levels up to 15 h (end point of experiment) (Bengtsson et al. 1996, Bengtsson et al. 2000).

Based on tissue distribution it can be discerned that the different receptor subtypes are involved in different physiological effects, e.g. the β1-ARs can act as vasodepressors, the β2-ARs are involved in bronchodilation, and the β3-ARs are involved in lipolysis (Strosberg 1997). In brown adipocytes, β-ARs are involved in regulation of gene expression, lipolysis, proliferation, differentiation and nonshivering thermogenesis (see further in section “mediators of signaling”) (Cannon and Nedergaard 2004).

The β-AR subtypes have been targeted to generate different βx-AR-ablated mice. The β1-AR-ablation (generated by (Rohrer et al. 1996)) can be embryonic lethal and those β1-AR-ablated mice that do survive have a loss of the chronotropic and inotropic response from agonist stimulation in the heart. The β2-AR-ablated mice (generated by (Chruscinski et al. 1999)) have a reduced vasodilation response, reduced respiratory change ratio, increased total exercise capacity, and exercise-induced hypertension. The β1/β2-AR-ablated mice (generated by (Rohrer et al. 1999)) have metabolic impairments, reduced effect of agonist stimulation but increased β3-AR agonist-induced effect. The β3-AR-ablated mice (generated by (Susulic et al. 1995)) have reduced body fat, reduced effect of agonist stimulation and higher expression of β1-AR mRNA (Hein and Kobilka 1997, Karasinska et al. 2003). Additionally, mice lacking all β-AR have been generated (Bachman et al. 2002, Jimenez et al. 2002). Compared to the wild-type mice, these so-called β-less mice were obese and cold-sensitive but did however display normal lipolytic responses to fasting. It was also concluded that β-AR signaling is required for diet-induced thermogenesis (Bachman et al. 2002, Jimenez et al. 2002).

The natural ligands for the β-AR are the sympathetic neurotransmitter noradrenaline and the hormone (and central neurotransmitter) adrenaline (Bylund et al. 1994, Docherty 1998). The affinities for the ligands are
somewhat different between the receptors; for the $\beta_1$-AR the ligands are equally potent, for the $\beta_2$-AR epinephrine is much more potent than norepinephrine, and for the $\beta_3$-AR norepinephrine is more potent than epinephrine (Bylund et al. 1994, Strosberg 1997). The $\beta_1$-AR are also sometimes stated to be more sensitive to NE than the $\beta_3$-AR (Cannon and Nedergaard 2004); however, in brown adipose tissue no difference could be seen in the response to NE in preadipocytes (containing $\beta_1$-AR) compared to mature adipocytes (containing $\beta_3$-AR) (Bronnikov et al. 1999).

Selective agonists and antagonists against the $\beta$-AR exist. Discussed below in the context of the experiments are the $\beta$-AR agonist isoprenaline, the $\beta_3$-AR selective agonists BRL37344 and CL316,243 and the $\beta_3$-AR selective agonist (and $\beta_1$- and $\beta_2$-AR specific antagonist) CGP12177. Also antagonists exist for $\beta_1$-AR (CGP20712), $\beta_2$-AR (ICI118551) and $\beta_3$-AR (SR59230, and L748337).

**Mediators of signaling**

The $\beta$-ARs are able to couple to stimulatory G-proteins ($G_s$-protein) and thereby activate adenyl cyclase (AC) to convert ATP to cyclic AMP (cAMP) (Gilman 1987, Chaudhry et al. 1994). However, the receptors are also able to couple to inhibitory G-proteins ($G_i$-proteins) (Daaka et al. 1997, Zou et al. 1999, Baillie et al. 2003). The $\beta_3$-ARs have been shown to be able to interact with both $G_s$ and $G_i$ (Chaudhry et al. 1994, Gauthier et al. 1996, Gerhardt et al. 1999, Soeder et al. 1999) although the splice variants seem to couple differently, with the $\beta_3a$-AR only coupling to $G_s$ and the $\beta_3b$-AR coupling to both $G_s$ and $G_i$ (Hutchinson et al. 2002). $\beta_3$-AR have been shown to also directly interact with the SH3 (Src homology 3) domain of Src and thereby activate Erk1/2 (and not via cAMP or PKA) (Cao et al. 2000); thus, the receptors may be able to signal without coupling to G-proteins.

The elevation of cAMP caused by $G_s$-coupling can lead to the activation of protein kinase A (PKA), Epac (exchange protein directly activated by cAMP, a Rap guanine nucleotide exchange factor) or other guanine nucleotide exchange factors (GEFs) (Kawasaki et al. 1998, de Rooij et al. 1998, de Rooij et al. 2000, Beavo and Brunton 2002, Sands and Palmer 2008). The activation of PKA by cAMP is able to activate Erk1/2 and the transcription factor Elk-1 through a PKA/Rap1/B-Raf-dependent pathway, where Rap1 is activated by PKA (Vossler et al. 1997). Other functions of PKA are to regulate the transcription factor cAMP-response-element-binding protein (CREB), the activating transcription factor 1 (ATF1) and the cAMP-responsive-element modulator (CREM). CREB is able to regulate gene expression by binding to CRE domains in the proximal promoter and distal enhancer regions of numerous genes, and the specific variant of CREM, ICER (inducible cAMP early repressor), can act as a negative feedback on CRE-mediated transcription. Epac can positively regulate transcription factors such as C/EBP$\beta$, CREB, and SOCS-3, and also c-Jun and ATF2 have
been implicated as targets (Beavo and Brunton 2002, Sands and Palmer 2008).

The level of cAMP varies throughout the cell, and as AC, PDE (phosphodiesterase), PKA and Epac signaling seems to be compartmentalized, it provides the possibility of obtaining different cAMP-dependent responses (Zaccolo et al. 2006, Lynch et al. 2007, Sands and Palmer 2008).

Termination of cAMP signaling is via the PDE that catalyse the hydrolysis of cAMP to 5’ AMP (Beavo and Brunton 2002, Sands and Palmer 2008).

Apart from inhibiting adenylyl cyclase, the coupling of β-AR to G\textsubscript{i}-proteins can lead to the activation of PI3K/Akt and subsequently Erk1/2 (not via G\textsubscript{s}-cAMP), and also signaling via a G\textsubscript{βγ}/Src/Ras/Raf-1-pathway (Zou et al. 1999, Gerhardt et al. 1999).

PKA and G-protein receptor kinases (GRKs, known earlier as βARK (β-adrenergic receptor kinase)) can phosphorylate β-AR and cause desensitization of the receptors. This is the case for both the β\textsubscript{1}- and β\textsubscript{2}-AR (Benovic et al. 1985, Benovic et al. 1986, Roth et al. 1991, Freedman et al. 1995, Strosberg 1997). Upon phosphorylation of the β-AR by GRK, β-arrestin is recruited (Lohse et al. 1992). Activated β-arrestin is a link to clathrin-dependent endocytosis (Lefkowitz 2004), and β-arrestin can recruit PDE (Perry et al. 2002), recruit Src (Luttrel et al. 1999), act as a signal transducer (Lefkowitz 2004, Lefkowitz and Shenoy 2005) and block the interaction with G\textsubscript{s}-proteins (Lohse et al. 1990). In contrast to the other β-AR, the β\textsubscript{3}-ARs have fewer sites for PKA- and GRK2-phosphorylation and these are in a non-favorable context (Liggett et al. 1993, Strosberg 1997). Instead, the regulation may occur on the mRNA level, since continuous β\textsubscript{3}-AR-agonist stimulation induces decreased receptor mRNA levels (although transiently) (Granneman and Lahners 1995, Klaus et al. 1995, Bengtsson et al. 1996) which could result in functional desensitization.

In brown adipose tissue, β\textsubscript{3}-AR can couple to G\textsubscript{s}-proteins and induce increased cAMP levels (see Fig. 10) (Zhao et al. 1997, Lindquist et al. 2000, Fredriksson et al. 2001, Cannon and Nedergaard 2004). β\textsubscript{3}-AR have also been shown to be able to couple to G\textsubscript{i}-proteins (Chaudhry et al. 1994, Gauthier et al. 1996, Gerhardt et al. 1999, Soeder et al. 1999); however, in mouse brown adipose tissue this seems not to be the case. Although PTX (a G\textsubscript{i}-protein inhibitor) can increase cAMP levels and augment β\textsubscript{3}-AR-induced cAMP levels, it seems that the G\textsubscript{i}-proteins are active regardless of which receptor is stimulated. Thus, G\textsubscript{i} might be inhibiting the cAMP formation in the basal cell state (Lindquist et al. 2000, Cannon and Nedergaard 2004). Since the splice variant β\textsubscript{3a}-AR is the variant mainly expressed in brown adipocytes, it also indicates that G\textsubscript{s}-coupling is the most relevant in these cells (Evans et al. 1999, Hutchinson et al. 2002).

cAMP is able to activate both PKA and Epac. However, in brown adipocytes there is as yet no indication that Epac is involved in any cAMP-mediated effects (Cannon and Nedergaard 2004). PKA is activated by NE in
brown adipocytes (see Fig. 10), and the PKA inhibitor H89 (which in these cells does not have a direct effect on the β3-ARs) can block all the effects of β3-AR-stimulation so far identified (Cannon and Nedergaard 2004).

Fig. 10. Adrenergic signaling in brown adipose tissue. See text for further information. The grey arrows indicate studies from laboratories that have seen different results from ours (β-AR to Erk1/2 and α2-AR to Erk1/2 (Valladares et al. 2000). Mediators that have not been confirmed as signaling intermediates are shown with grey-colored text (Rap-1, B-Raf, PIP2 and DAG).

The MAP kinase Erk1/2 can be activated via β3-AR in mouse brown adipocytes through a cAMP/PKA/Src-pathway (Lindquist et al. 2000). The small G-protein Rap-1 and the protein kinase B-raf are both expressed in mouse brown adipocytes (Lindquist and Rehnmark 1998). As they can be activated by PKA (see above), it is possible that they could also induce the Erk1/2 pathway (compare to (Vossler et al. 1997)). The activation of Erk1/2 does not involve Gi-proteins or PI3K, and forskolin can activate Erk1/2 via a PKA/Src-pathway (Lindquist et al. 2000). Also in rat brown adipocytes, NE, isoprenaline, and BRL-37344 were able to activate Erk1/2. Both BRL37344 and dibutyryl-cAMP were able to induce the same Erk1/2 levels as NE, thus indicating that the pathway to Erk1/2 was via β3/Gs/cAMP (PKC and PI3K were not involved in the signaling pathway) (Shimizu et al. 1997). In con-
In contrast to these studies, Valladares et al. found that in rat fetal brown adipocytes all adrenergic receptors were able to stimulate Erk1/2, but this was not through cAMP/PKA, and forskolin (agonist on AC) could not stimulate Erk1/2 phosphorylation (Valladares et al. 2000). For the pathway of β-adrenergic receptor signaling to Erk1/2 in brown adipocytes, see Fig. 10.

The activation of Erk1/2 by GPCR has also been proposed to be via transactivation of receptor tyrosine kinases such as EGFR and PDGFR. However, in brown adipocytes NE and CL316243 did not activate Erk1/2 via EGFR, and neither was the EGFR phosphorylated when stimulated with NE (Lindquist 2001); thus, in NE-induced Erk1/2 activation, transactivation of the EGFR is not an obligatory step.

The other two MAP kinases, p38 and JNK have also been investigated. p38 MAPK is activated via a β3-AR/PKA pathway (Cao et al. 2001) and as discussed in the review by Cannon and Nedergaard 2004, activation of the stress-activated JNK pathway is seen in brown adipose tissue in vivo during cold exposure, but in cell cultures NE does not stimulate this pathway (unpublished observations) (Cannon and Nedergaard 2004).

As mentioned above, PKA can also mediate the phosphorylation of CREB from adrenergic stimulation via a β-AR/cAMP-pathway (neither Src nor Erk1/2 are involved in this pathway). CREB can, however, also be phosphorylated via an α1-AR pathway (Thonberg et al. 2002). The increase in mRNA levels of ICER in brown adipocytes is via a β-AR/cAMP/PKA-pathway and does not occur via α1-AR/Ca2+/PKC (Thonberg et al. 2001).

The signaling via β3-AR/cAMP/PKA activates glucose uptake via a PI3K/conventional or novel PKC-pathway (Chernogubova et al. 2004). Although this mechanism is dependent on β3-AR in wild-type cells, the signaling is rescued by α1- and β1-AR in primary cultures from β3-AR-ablated mice (Chernogubova et al. 2005). Also, β-AR, via cAMP, stimulate AMPK, and inhibition of AMPK blocks the β-AR-stimulated glucose uptake partially (Hutchinson et al. 2005). β3-AR-stimulation leads to increased expression of GLUT1 but decreased expression of GLUT4. The glucose uptake induced by β3-AR seems to act via two mechanisms, one not dependent on de novo synthesis of GLUT1 or 4 (or on GLUT1 and GLUT4 translocation), and one dependent on newly synthesized GLUT1 protein and increase of GLUT1 at the plasma membrane (Dallner et al. 2006).

UCP1 expression is regulated by β-AR signaling. In preadipocytes, UCP1 mRNA expression is not induced by NE (Cannon and Nedergaard 2004). However, in mature brown adipocytes, NE, BRL37344, CGP-12177, forskolin, and 8-Br-cAMP all increase UCP1 mRNA levels; thus, UCP1 mRNA can be activated by a β3-AR/cAMP-pathway. Furthermore, the β3-AR-induced UCP1 mRNA expression is mediated by PKA (Rehnmark et al. 1990, Fredriksson et al. 2001). In fetal rat brown adipocytes, UCP1 expres-
sion was dependent on a β-AR/cAMP/PKA-pathway, which was independent of Erk1/2 phosphorylation (Valladares et al. 2000). Thus, the MAP kinase Erk1/2 is not involved in regulating the UCP1 mRNA expression. However, p38 MAP kinase can induce UCP1 expression (via a cAMP/PKA pathway) (Cao et al. 2001).

Other genes influenced by β-AR signaling are c-fos (via cAMP but also Ca^{2+}) (Thonberg et al. 1994) and VEGF (via a β₁/β₃-AR/cAMP/PKA/partly Src-pathway (not Erk1/2)) (Fredriksson et al. 2000).

In brown adipocytes, norepinephrine can stimulate proliferation via β₁-AR (in immature adipocytes) and stimulate differentiation (e.g. UCP1 gene expression) via β₃-AR (in confluent adipocytes). This shift in use of receptors can be seen over time in brown adipocyte cultures. Both receptors mediate their response via cAMP (Bronnikov et al. 1992, Bronnikov et al. 1997, Bronnikov et al. 1999). However, in fetal rat brown adipocytes, cell growth is mediated via β-AR/Erk1/2 and is not dependent on either cAMP or PKA (Valladares et al. 2000).

In brown adipocytes, norepinephrine and isoprenaline can inhibit apoptosis (promote cell survival) via β-AR/cAMP/PKA/Src/Erk1/2. Mice exposed to cold have reduced apoptosis in their brown adipose tissue (Lindquist and Rehnmark 1998, Cannon and Nedergaard 2004). This contributes to the increase in cell numbers in the tissue after cold acclimation.

**Interaction with caveolin and caveolae**

The β₃-ARs have been shown to cofractionate with Cav1 in 3T3-L1 adipocytes and to colocalize with Cav1 in mouse small intestine (El-Yazbi et al. 2006, Ahmad et al. 2009). In adipocytes, stimulation with the β₃-agonist CL316,243 did not influence the localization of the β₃-AR (Ahmad et al. 2009). β₁- and β₂-AR colocalization with caveolin has also been investigated. In COS-7 cells overexpressing either β₁-AR or β₂-AR, both cofractionated with Cav1 (Schwencke et al. 1999), and in both adult and neonatal rat cardiomyocytes, β₂-AR colocalized with Cav3 (Rybin et al. 2000, Rybin et al. 2003). This is in contrast to what is seen in HEK293 stably transfected with β₂-AR and transiently transfected with Cav3, where no colocalization could be seen (Pontier et al. 2008). All of these studies have used fractionation as their main method, and as discussed under methods, the use of fractionation (dependent on method though) cannot conclude whether the receptors are truly in caveolae or if they reside in the lipid raft fraction. Thus, a delineation of whether the β-ARs are localized to caveolae or not, is not possible with these studies. It should be stated though that the β₃α₃-AR have a putative caveolin-binding site, and when this site is mutated, the β₃α₃-AR can interact with both Gₛ and Gₛ-proteins, thus implying that caveolin is involved in regulating β₃α₃-AR signaling (Sato et al. 2007).

The expression of β₃-AR in the Cav1-ablated mice has given varying results depending on the tissue investigated; in perigonadal fat pads there was
no difference in the protein levels of β3-AR (Cohen et al. 2004); nor in the mRNA levels in the saphenous artery (Neidhold et al. 2007). Similarly, in 3T3-L1 cells treated with Cav1 siRNA there was no effect on β3-AR protein levels (Ahmad et al. 2009). In contrast, the Cav1-ablated mice had less β3-AR mRNA in murine detrusor (Wuest et al. 2009), and as shown in paper I, lower β3-AR protein levels in brown adipose tissue. An interesting finding, as discussed above in section 2.1, is that chronic β-adrenergic receptor stimulation in mice leads to downregulation of caveolin (Oka et al. 1997). Thus, caveolin may affect β-AR expression depending on tissue, but the β-AR pathway can also regulate caveolin expression.

The influence of Cav1 on relaxation via β-ARs in mouse small intestine, saphenous artery, and murine detrusor has been investigated (El-Yazbi et al. 2006, Neidhold et al. 2007, Wuest et al. 2009). In the small intestine of Cav1-ablated mice, the isoprenaline-stimulated relaxation was desensitized, and the basal was higher than in the wild-type small intestine. Inhibition of the different β-AR subtypes (CGP20712A (β1), ICI118551 (β2) and SR59230 (β3)) shifted the relaxation curves to the right in the wild-type intestine but did not shift them further in the Cav1-ablated mice; thus indicating that all the β-AR signaling was dependent on Cav1. Additionally, the β3-AR-agonist BRL37344-stimulated relaxation in the Cav1-ablated mice was also desensitized (El-Yazbi et al. 2006). In contrast, in the saphenous artery, Cav1-ablation had no effect on β2-AR-induced relaxation, while β1-AR-induced relaxation was abolished and instead a β3-AR-induced relaxation appeared. Thus, the β2-AR effect did not require Cav1, while the β1-AR effect required Cav1, and the β3-AR effect was inhibited by Cav1 (Neidhold et al. 2007). The two studies above are also in contrast to the ones in murine detrusor, where in wild-type detrusor only β2-AR seems to respond to isoprenaline (antagonists against β1- and β3-AR (CGP20712 and L748,337, respectively) do not cause a shift to the right as does the β2-AR antagonist (ICI118,551) after isoprenaline-stimulated relaxation), and in detrusor from Cav1-ablated mice, both antagonists against β2- and β3-AR partially shifted the relaxation curve (no effect of β1-AR) (Wuest et al. 2009). Thus, the β2-AR signaling is strengthened by Cav1, while the β3-AR signaling is inhibited by Cav1. Wuest et al. also show that relaxation in human detrusor is only dependent on the β3-AR (Wuest et al. 2009). In conclusion, the β3-AR relaxation is desensitized in Cav1-ablated mouse small intestine, and in the saphenous artery and the murine detrusor, β3-AR relaxation is inhibited by Cav1. Thus, depending on the tissue, Cav1 seems to have different roles in β3-AR-mediated relaxation.

As already discussed in section 4.2, the effect of Cav1-ablation on β3-AR signaling in white adipocytes has also been studied. Cohen et al. showed that in wild-type perigonadal fat pads, the β3-agonist CL316,243 (CL) induced a complex formation between perilipin, Cav1 and PKAc, that did not form in
Cav1(-/-) fat pads (the complex is also formed in 3T3-L1 cells). The Cav1(-/-) fat pads had increased protein levels of the PKA regulatory subunits RI and RIIa, and the PKA activity was higher. After stimulation with CL, the phosphorylation of cAMP response element binding protein (CREB) was increased, while the phosphorylation of perilipin was reduced (Cohen et al. 2004). In agreement, knock-down of Cav1 in 3T3-L1 cells inhibits the CL-induced phosphorylation of HSL and perilipin (Ahmad et al. 2009). Thus, Cav1 is important in β3-adrenergic signaling to PKA and downstream targets.

Activation of β3-adrenergic receptors can induce cAMP. In C6 glioma cells transfected with Cav1 siRNA, Allen et al. found that the isoprenaline-induced cAMP levels were increased compared to the levels in wild-type cells (Allen et al. 2009). Thus, caveolin has a negative effect on β-AR-stimulated cAMP accumulation in these cells. In contrast, in Cav1(-/-) mature brown adipocyte cultures, stimulation with the specific β3-agonist CL316,243 (CL) did not increase the cAMP levels to the same level as in the wild-type cultures (see paper IV). Also the adenylyl cyclase-agonist forskolin gave a lower cAMP level in the Cav1(-/-) cultures, although not to the same levels as the β3-agonist reduced levels. Thus, the CL-induced signaling to cAMP in brown adipocytes is dependent on Cav1.

Treatement of brown adipocytes with the cholesterol-disturbing agent filipin leads to reduced CL-stimulated cAMP accumulation (Sato et al. 2007), thus indicating a positive role of membrane rafts in β3-AR signaling. Furthermore, pre-treatment with PTX was able to rescue the signal (Sato et al. 2007), thus Gs-coupling was responsible for the reduction in cAMP levels.

The role of Cav1 for the activity of phosphodiesterases (PDE) has been investigated. Ahmad et al., found that in 3T3-L1 cells, CL preferentially activated phosphodiesterase 3B (PDE3B) that was associated with Cav1. Cav1 siRNA knockdown reduced the amount of CL-induced PDE3B activity. Also in epididymal fat pads from Cav1-ablated mice, PDE3B activity was decreased and the CL-induced activation of PDE3B was inhibited (in comparison to the insulin-induced response that was not affected) (Ahmad et al. 2009). Thus, not only cAMP formation is impaired by Cav1-ablation, but also PDE3B activity is impaired (see also section 4.2). In conclusion, Cav1 can affect signaling from β-ARs; this effect seems to be tissue-specific and to depend on which receptor subtype that is investigated. Cav1 seems to play a role in the β3-AR regulation of lipolysis in both white and brown adipose tissue.
5.2 α2-adrenergic receptors

The α2-adrenergic receptors are divided into three subtypes: α2A/D, α2B and α2C (Docherty. 1998). The receptors are expressed in many different tissues; however, the expression of the different subtypes is variable between tissues (Saunders and Limbird 1999). The α2-adrenergic receptors are also expressed in brown adipose tissue (Cannon and Nedergaard 2004). In rat white pre-adipocytes, the agonist UK14304 stimulates proliferation (Bouloumie et al. 1994), while in fetal rat brown adipocytes, α2-ARs were able to inhibit cell proliferation (Valladares et al. 2000).

Different α2-AR-ablated mice have been generated. Two different α2A-AR-ablated mice have been generated, one on a 129xC57Bl/6 background (MacMillan et al. 1996) and the other one on a 129SvxFVB/N (Altman et al. 1999). MacMillan et al. found a loss of agonist hypotensive response, and Altman et al. found increased sympathetic activity and a reduced amount of cardiac β-ARs (MacMillan et al. 1996, Altman et al. 1999). α2B-AR-ablated mice (generated by (Link et al. 1996)) show loss of agonist hypertensive response, and α2C-AR-ablated mice (generated by (Link et al. 1995)) are viable and fertile and appear grossly normal. The double-α2A/α2C-AR-ablated mice (generated by (Hein et al. 1999)) lack presynaptic control of NE release, have increased plasma NE levels, develop cardiac hypertrophy and have reduced ventricular contractility (Karasinska et al. 2003).

The natural ligands for the α2-ARs are, as for the β-AR, norepinephrine and epinephrine (Bylund et al. 1994, Docherty 1998). Selective agonists and antagonists against the α2-AR exist. Discussed below are the α2-AR agonist clonidine (above also UK14304) and the antagonist yohimbine.

Mediators of signaling

α2-ARs are able of coupling to G-proteins. The common pathway for the receptors is via G1-coupling and the inhibitory effect of the Gαi-subunit on adenyllyl cyclase (i.e. lower production of cAMP) (Docherty 1998). However, also the βγ-subunit has been implicated in mediating α2-AR signaling (Clapham and Neer 1997), e.g. by activating MAP kinase (Faure et al. 1994, Lopez-Iliasaca et al. 1997).

Other implicated mediators of the signaling pathway(s) are Src, Pyk2, Erk1/2, PLA2, PLC, PLD, and the receptors can activate receptor-operated K+channels and inhibit voltage-gated Ca2+-channels (Alblas et al. 1993, Della Rocca et al. 1997, Saunders and Limbird 1999). Furthermore, stimulation of α2-AR leads to the release of LPA in human white adipocytes (see LPA section) (Valet et al. 1998).

Protein kinase C (PKC), G-protein coupled protein kinase (GRK), and the scaffolding protein spinophilin have been shown to interact with the α2-ARs. PKC is able to phosphorylate the receptor and are implicated in mediating
heterologous desensitization, and may affect coupling to G-proteins and thereby the activity of the receptors. GRK2 has been shown to phosphorylate the receptor and is implicated in homologous desensitization of the receptors. GRK2 phosphorylation recruits arrestins, which can stabilize the $\alpha_2$-AR phosphorylation and mediate receptor endocytosis and thereby recycling. Arrestin may play a role in resensitization of the receptor and also in localization of $\alpha_2$-AR-stimulated Erk1/2 (Saunders and Limbird 1999, Wang and Limbird 2007). Additionally, $\alpha_2$-AR-stimulated Erk1/2 activation via Src seems to be dependent on arrestin. The scaffolding protein spinophilin, seems to counteract the effect of arrestin and leads to reduced receptor phosphorylation, signaling desensitization and receptor internalization (Wang and Limbird 2007).

In mature mouse brown adipocytes, stimulation with norepinephrine at higher concentrations (≥0.1 μM) in the presence of yohimbine ($\alpha_2$-AR antagonist) increased cAMP levels to levels comparable with selective stimulation with $\beta_3$-AR specific agonists (Bronnikov et al. 1999). Thus, $\alpha_2$-AR inhibit the increase in cAMP seen with NE-stimulation. G$_i$-proteins are present in mouse brown adipocytes (Svoboda et al. 1996, Bourova et al. 2000), and the G$_i$-protein inhibitor pertussis toxin (PTX) was shown to increase both BRL37344 ($\beta_3$-agonist)- and ACTH-stimulated cAMP levels (Lindquist et al. 2000). Thus, a G$_i$-protein is also present that can inhibit cAMP production. This G$_i$-protein was however not required for Erk1/2 phosphorylation in response to BRL37344 or NE (Lindquist et al. 2000). Direct stimulation of $\alpha_2$-ARs with clonidine did not increase Erk1/2 phosphorylation (Lindquist 2001). In conclusion, these data indicate that $\alpha_2$-AR in brown adipocytes is able to inhibit cAMP production via a G$_i$-protein, and that the $\alpha_2$-AR cannot activate Erk1/2. However, Valladeres et al. showed that in fetal rat brown adipocytes, $\alpha_2$-ARs were able to stimulate Erk1/2 phosphorylation. The $\alpha_2$-ARs were also able to inhibit cell proliferation, and did not influence the UCP1 mRNA levels (Valladares et al. 2000). In Fig. 10, part of the signaling mediators of $\alpha_2$-AR in brown adipose tissue is summarized.

*Interaction with caveolin and caveolae*

So far, only one study has investigated the importance for caveolae/lipid rafts for $\alpha_2$-ARs. Ollé-Láhdesmäki et al. investigated the internalization mechanism after NE stimulation in PC12 cells and HEK293 cells, with both cell lines stably expressing either human $\alpha_{2A}$- or human $\alpha_{2B}$-AR. They utilized hyperosmotic sucrose pretreatment to block clathrin-dependent endocytosis and filipin to block formation of caveolae from the plasma membrane. They found that the $\alpha_{2B}$-AR seems to only be dependent on clathrin-dependent endocytosis, while the $\alpha_{2A}$-AR seemed to utilize both clathrin-dependent and caveolae-mediated endocytosis (Olli-Lahdesmaki et al. 2003). However, since filipin works as a cholesterol-binding agent, not only caveolae will be affected but also lipid rafts and other endocytotic pathways. Thus, the con-
clusion should be that $\alpha_{2A}$-AR utilize both clathrin-dependent and filipin-sensitive endocytosis.

5.3 $\alpha_1$-adrenergic receptors

The $\alpha_1$-adrenergic receptors can be divided into three subtypes: $\alpha_{1A}$, $\alpha_{1B}$, and $\alpha_{1D}$. Possibly, also a fourth subtype exists, the $\alpha_{1L}$ (Hieble et al. 1995, Garcia-Sainz et al. 1999, Docherty 1998). The $\alpha_1$-ARs are expressed in many cell types; however, the expression of the different subtypes is very variable between both organs and species (Garcia-Sainz et al. 1999).

In brown adipose tissue, $\alpha_1$-ARs are present, with the $\alpha_{1A}$-AR being the primarily expressed (Cannon and Nedergaard. 2004). Kikuchi-Utsumi et al. showed that in rats acutely exposed to cold, the expression of $\alpha_{1A}$-AR was increased, the $\alpha_{1B}$-AR was unchanged, and $\alpha_{1D}$-AR was transiently decreased in the brown adipose tissue (also seen with norepinephrine). Long-term acclimation to cold resulted in an up-regulation of the $\alpha_{1A}$-AR, and down-regulation of the $\alpha_{1B}$- and $\alpha_{1D}$-AR (Kikuchi-Utsumi et al. 1997). However, in HEK293 cells stably expressing the different $\alpha_1$-AR subtypes, Lei et al showed that stimulation with norepinephrine down-regulated $\alpha_{1A^\text{ antagonist}}$ and $\alpha_{1D}$-AR density, but the $\alpha_{1B}$-AR density was increased (Lei et al. 2001).

Although $\beta_3$-ARs are predominantly responsible for lipolysis and nonshivering thermogenesis in brown adipocytes, the $\alpha_1$-ARs can also contribute to a small extent (Cannon and Nedergaard 2004), and e.g. $\alpha_1$-ARs can increase the thermogenic effect of $\beta_3$-AR-generated cAMP in Syrian hamster brown adipocytes (Zhao et al. 1997).

$\alpha_1$-AR-ablated mice have been generated with regard to the different $\alpha_1$-AR subtypes (for further information see (Sanbe et al. 2009)) as well as an $\alpha_1$-AR triple-ablated mouse ($\alpha_{1A^\text{ antagonist}}, \alpha_{1B^-}$ and $\alpha_{1D}$-ablated) (Sanbe et al. 2007, Sanbe et al. 2009). However, none of these studies has investigated the effect on brown adipose tissue in these mice. In the triple-ablated mice, it was shown that $\alpha_1$-AR was important for normal contractility of the vas deferens and fertility (Sanbe et al. 2007). The triple-ablated mice also have enhanced vascular contractility in the thoracic aorta (Sanbe et al. 2009).

In the C-terminal parts of the receptor there are sites that can be phosphorylated by several protein kinases, e.g. protein kinase C (PKC), G-protein receptor kinase (GRK) and protein kinase A (PKA). These phosphorylations can be involved in receptor internalization and desensitization (Garcia-Sainz et al. 1999).

The natural ligands for the $\alpha_1$-ARs are as for the $\alpha_2$-ARs and the $\beta$-ARs norepinephrine and epinephrine (Bylund et al. 1994, Docherty 1998). Selective agonists and antagonists against the $\alpha_1$-AR exist. Discussed below is the $\alpha_1$-AR agonist cirazoline.
Mediators of signaling

The α1-ARs have been shown to couple to G_{q/11}-proteins. This leads to the activation of phospholipase C (PLC), which in turn cleaves phosphatidylinositol 4,5-bisphosphate PI(4,5)P2 to diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG can activate protein kinase C (PKC), and IP_3 triggers the release of Ca^{2+} from the endoplasmic reticulum leading to an increase in intracellular Ca^{2+} (Wu et al. 1992, Wu et al. 1995, Docherty 1998, Garcia-Sainz et al. 1999, Hubbard and Hepler 2006). However, the receptors have also been shown in certain systems to couple to G_s, G_i, G_o and G_{12/13} (Gudermann et al. 1997, Docherty 1998, Garcia-Sainz et al. 1999, Khorchid et al. 1999, Melien et al. 2000, Gallego et al. 2005, Hein and Michel 2007). The G_s-pathway leads to an increase in cAMP levels and an activation of PKA, and the G_i-pathway has been shown to lead to PI3K and Ras/Raf/MEK/MAPK signaling (Garcia-Sainz et al. 1999, Khorchid et al. 1999, Gallego et al. 2005). Other implicated mediators of α1-ARs signaling are Rho (Sah et al. 1996), Src, PYK2 (Della Rocca et al. 1997), PLA_2, PLD (Hein and Michel 2007), JNK and p38 (Yamauchi et al. 2001). Additionally, in L6 muscle cells, the α_{1A}-AR is able to activate glucose uptake in a PLC-, PI3K-, and atypical PKC-dependent manner (Hutchinson and Bengtsson 2005). Furthermore, the α_1-ARs can regulate the gene expression of the early genes c-jun and c-fos (Garcia-Sainz et al. 1999).

In brown adipose tissue, cirazoline has been shown to reduce apoptosis and induce Erk1/2 activation (Lindquist and Rehnmark 1998). This Erk1/2 activation was via Src, but did not involve G_{i}-proteins, PI3K, or PKA (Lindquist et al. 2000). Stimulation of α_{1A}-ARs also leads to the formation of IP_3 (Nanberg and Putney 1986) and increases intracellular Ca^{2+} levels (Wilcke and Nedergaard 1989). This is probably through G_{q}-proteins, which are present in brown adipose tissue (shown by (Bourova et al. 2000)). The increase in Ca^{2+} levels activates phosphodiesterase (PDE) that is able to break down cAMP during forskolin treatment (Bronnikov et al. 1999). However, direct stimulation of the α_{1A}-ARs did not affect the cAMP levels (Thonberg et al. 1994). Stimulation of α_{1A}-ARs also leads to CREB phosphorylation via PKC (no involvement of Ca^{2+}) (Thonberg et al. 2002), and induces gene expression of c-fos (Thonberg et al. 1994), but has no effect on VEGF mRNA levels (Fredriksson et al. 2000). In Fig. 10, part of the signaling mediators of α_1-AR in brown adipose tissue is summarized.

Interaction with caveolin and caveolae

The α_{1A}-ARs have been indicated to cofractionate with caveolins in CHO cells (hamster α_{1B}-ARs cofractionated with Cav1) (Toews et al. 2003), and shown to cofractionate in rat cardiac tissue (Cav3) (Fujita et al. 2001), and in rat heart membrane (Cav3, only 25% colocalize) (Lanzafame et al. 2006). However, in rat tail artery (Cav1) (Dreja et al. 2002) and adult mouse cardiac myocytes from α_{1A}/α_{1B}-AR-ablated mice transfected with either α_{1A}-AR-
GFP or $\alpha_{1B}$-AR-GFP (Cav3) (Wright et al. 2008), the receptors did not colocalize with caveolin. Thus, whether the receptors colocalize to caveolin might be dependent on the cell type or the methodology used. The studies have used fractionation as their main method and as discussed above, this does not conclude that the receptors are actually in caveolae compared to lipid rafts. However, Fujita et al. used immunoprecipitation (Cav1 and $\alpha_{1}$-AR colocalized) and Wright et al. used confocal microscopy (did not colocalize); thus these possibly more specific methods (depending on antibody specificity and methodology) did not shed more light on the localization of the receptors.

The effect on $\alpha_{1}$-AR signaling in Cav1-ablated mice has been investigated. Neidhold et al. found that there was no difference in the mRNA expression levels of $\alpha_{1A}$-, $\alpha_{1B}$- or $\alpha_{1D}$-AR in the saphenous artery of Cav1-ablated mice compared to levels in the wild-type mice, and no difference in $\alpha_{1}$-AR-mediated contractions (Neidhold et al. 2007). Albinsson et al. found also no differences in the $\alpha_{1}$-AR-mediated contractions in the aorta of Cav1-ablated mice compared to $\alpha_{1}$-AR-mediated contractions in the wild-type mice. However, in the small mesenteric arteries there was an increase in the $\alpha_{1}$-AR-mediated contractions (Albinsson et al. 2007). Furthermore, Shakirova et al. found that femoral arteries in Cav1-ablated mice have higher contraction force after cirazoline stimulation (Shakirova et al. 2006). Thus, depending on the tissue, Cav1 seems to be important for $\alpha_{1}$-AR-mediated contraction.

Methyl-$\beta$-cyclodextrin (m$\beta$CD) has been widely used to investigate the influence of caveolae/lipid rafts on $\alpha_{1}$-AR signaling. Dreja et al. found that the $\alpha_{1}$-AR-mediated contraction in rat tail artery was not changed after treatment with m$\beta$CD (Dreja et al. 2002). In contrast, Morris et al. found that in neonatal rat cardiomyocytes, m$\beta$CD inhibited the rise in the inositol phosphate levels after phenylephrine stimulation (Morris et al. 2006), and Wang et al. found that cat atrial myocytes treated with m$\beta$CD failed to increase NO$_i$ after phenylephrine stimulation (Wang et al. 2005). Thus, in these two studies, caveolae/lipid rafts seem to be of importance for $\alpha_{1}$-AR signaling. A third scenario has been seen in rat-1 fibroblasts stably transfected with human HA-$\alpha_{1a}$-AR, where Lei et al. found that treatment with m$\beta$CD increased the basal levels of inositol phosphates and that after phenylephrine treatment a lower maximum was obtained (Lei et al. 2009). Thus, caveolae/lipid rafts inhibited the basal levels, but were needed for the $\alpha_{1a}$-AR-mediated response. In these cells, m$\beta$CD also altered the binding affinity for phenylephrine (increased) and prazosin (decreased), and the level of $\alpha_{1a}$-AR on the cell surface was decreased (Lei et al. 2009). Thus, also here, depending on tissue/cell type, caveolae/lipid rafts (cholesterol) seem to have different impacts on $\alpha_{1}$-AR-signaling.
In addition, the agent filipin has been utilized. Wright et al. found that filipin treatment reduced the amount of phosphorylated Erk1/2 that colocalized to Cav3 after phenylephrine stimulation (α1A/α1B-AR knockout adult mouse cardiac myocytes with transfected α1A-GFP) (Wright et al. 2008).

Signaling mediators of the α1-AR pathway have also been shown to colocalize with Cav3; e.g. Gq, PLCβ1, PLCβ3 and inositol phosphates (PI, PIP and PIP2) (Fujita et al. 2001, Lanzafame et al. 2006, Morris et al. 2006).

In conclusion, caveolin and caveolae might play a role in localization of the α1-AR and its signaling. Whether this is dependent on cell type or methodology remains to be seen.

5.4 LPA receptors
The lysophosphatidic receptors (LPAxR) are G-protein-coupled receptors (GPCRs) that are expressed in most investigated cell types. They can be divided into two structurally different groups, the endothelial differentiation gene (Edg) receptor family, and the “non-Edg” receptor family. The Edg family consists of LPA receptor 1, 2 and 3 (Edg2/Vzg-1, Edg4 and Edg7, respectively) and the non-Edg family of LPA receptor 4, 5 and 6 (p2y9/GPR23, GPR92/GPR93 and p2y5, respectively) (Anliker and Chun 2004, Moolenaar et al. 2004, Ishii et al. 2009). In Fig. 11, a phylogram of the mouse LPA receptors and the related Edg-family receptors for sphingosine-1-phosphates (S1Ps) is shown.

Fig. 11. Phylogram of LPA receptors. The nucleotide sequences were taken from the nucleotide database in NCBI (National Center for Biotechnology Information), LPA1R: NM_010336.2, LPA2R: NM_020028.3, LPA3R: NM_022983.4, LPA4R: NM_175271.4, LPA5R: NM_001163268.1, LPA6R: NM_175116.4, S1P1R: NM_007901.5, S1P2R: NM_010333.4, S1P3R: NM_010101.4, S1P4R: NM_010102.1 and S1P5R: NM_053190.2. The phylogram was generated in the program ClustalW2.

LPAxR-ablated mice have been generated. LPA1R-ablated mice (generated by (Contos et al. 2000)) have reduced body size, reduced brain mass, show craniofacial dysmorphism, defects in the cortical development, and impaired suckling behavior. The mice show 50% perinatal lethality. LPA2R-ablated mice show no abnormalities and double-LPA1R/LPA2R-ablated mice
have the same phenotype as the LPA_1R-ablated mice (both generated by 
(Contos et al. 2002)). LPA_3R-ablated mice (generated by (Ye et al. 2005))
show delayed implantation, altered embryo spacing, and reduced litter size 
(Choi et al. 2008). Regarding the other LPA-R subtypes, no ablated mice 
have been generated as yet.

Lysophosphatidic acid (LPA, 1- or 2-acyl-sn-glycero-3-phosphate) is a 
bioactive phospholipid that plays important roles in various biological 
processes, such as proliferation, cell survival, cell motility, and differentia-
component in these processes is the Erk1/2 MAP kinase pathway (Dixon and 
be secreted from cell types such as platelets, fibroblasts, adipocytes and can-
cer cells, is present in e.g. serum and plasma, and can act in both an auto-
crine and a paracrine manner (Pages et al. 2001b). Secretion of LPA from 
human adipocytes has been shown to be under the regulation of α_2-AR sti-
mulation, and both conditioned medium and LPA by itself can regulate cell 
spreading and proliferation of the adipocyte-like cell line 3T3-F442A (Valet 
et al. 1998).

**Mediators of signaling**

As the LPA receptors belong to the G-protein coupled receptor family, 
they can couple to G-proteins. The receptors are able to couple to any of the 
four types of G-proteins: G_1/o, G_q/11, G_12/13 and G_s (Moolenaar 1999, Anliker 
and Chun. 2004), with LPA_1R and LPA_3R coupling to G_1/o, G_q/11 and G_12/13, 
LPA_2R to G_1/o and G_q/11, LPA_4R to G_i, G_q/11, G_12/13, and G_s, LPA_5R to G_q/11 
and G_12/13, and LPA_6R to G_i, G_12/13, and G_s as yet (depicted in Fig. 12) (Ishii 
et al. 2009).

![Fig. 12. G-coupling of LPA receptors.](image)

Further mediators of the LPA signaling pathway are phosphatidylinositol-3-
kinase (PI3K), protein kinase C (PKC), and Src. PI3K can mediate signaling

The LPA-induced Erk1/2 activation is generally thought to be activated via so-called transactivation, and several articles have reported on this phenomenon (Daub et al. 1996, Cunnick et al. 1998, Herrlich et al. 1998, Della Rocca et al. 1999, Goppelt-Struebe et al. 2000, Kue et al. 2002, Wang et al. 2003, Shah et al. 2006, Xu et al. 2007, Rodland et al. 2008). It has been shown that the transactivation can be mediated via epidermal growth factor (EGF) receptors (Daub et al. 1997, Gschwind et al. 2002, Kue et al. 2002, Shah et al. 2005, Xu et al. 2007, Rodland et al. 2008) or platelet-derived growth factor (PDGF) receptors (Herrlich et al. 1998, Goppelt-Struebe et al. 2000). However, in some cell types investigated, the dependence on EGF (or PDGF) receptor transactivation is only partial (Della Rocca et al. 1999, Goppelt-Struebe et al. 2000, Wang et al. 2003, Shah et al. 2005, Gesty-Palmer et al. 2005). As discussed in paper III, in more physiological systems (i.e. primary cell cultures) it seems that transactivation is not as important for LPA signaling (Shah et al. 2005, Karagiosis et al. 2009). We show in paper III that in brown pre-adipocytes and the adipocyte-like cell line 3T3-F442A, LPA-induced Erk1/2 activation was independent of transactivation via the EGFR (also via the PDGFR). However, LPA-induced Erk1/2 activation in the transformed brown-fat derived cell line HIB-1B was dependent on EGFR transactivation. As further shown in paper III, brown pre-adipocytes express the LPA receptors 1-4 and 6, and LPA induced Erk1/2 activation via two non-transactivational pathways: one G_2-protein-PKC-Src dependent, and the other, a PTX-insensitive pathway, involving PI3K (but not Akt) activation. LPA did not induce cAMP, indicating that the activated LPA receptors are not G_2-coupled. In Fig. 13, the LPA signaling pathways in brown adipose tissue are depicted.

LPA has been shown to directly interact with peroxisome proliferator-activated receptor γ (PPARγ). McIntyre et al. showed that LPA is able to displace the PPARγ agonist rosiglitazone and that LPA could stimulate the expression of a PPAR-responsive element (PPRE) reporter (McIntyre et al. 2003). In white pre-adipocytes and 3T3-F442A cells, LPA has anti-adipogenic effects and induces proliferation, and Simon et al. showed that LPA reduced PPARγ2 gene expression (Valet et al. 1998, Pages et al. 2001a, Simon et al. 2005). Also in brown pre-adipocytes, LPA was found to reduce
PPARγ gene expression, pointing to dedifferentiating/pro-proliferative effect of LPA (unpublished observations).

Fig. 13. LPA signaling in brown adipose tissue.

**Interaction with caveolin and caveolae**

Not many studies have investigated the effect of caveolin on LPA signaling. In pCMVECs (porcine cerebral microvascular endothelial cells), Gobeil et al. showed that both clathrin and caveolin-1 (Cav1) immunoprecipitated with LPA1R at the plasma membrane, but at the nuclei only Cav1 co-precipitated, thus implying that LPA receptors are only internalized via caveolae and not clathrin. When these cells were treated with either filipin or methyl-β-cyclodextrin, there was no change in LPA-induced Cox-2 mRNA expression (Gobeil et al. 2003). Thus, caveolae/lipid rafts were not necessary for LPA signaling to increase Cox-2 mRNA expression. In Cos-7 cells, Kranenburg et al. showed that treatment with methyl-β-cyclodextrin decreased LPA-induced Erk1/2 phosphorylation, but increased LPA-induced ras activation (Kranenburg et al. 2001). So in contrast to Gobeil et al. (where Cox-2 mRNA expression was not changed), Kranenburg et al. showed that the presence of caveolae/lipid rafts activated LPA-induced Erk1/2 phosphorylation but inhibited ras activation. Sphingosine-1-phosphate (S1P)-signaling has also been investigated; Igarashi et al. found that the EDG-1 receptor co-immunoprecipitated with Cav1 and that overexpression of Cav1 diminished S1P-mediated endothelial nitric oxide synthase (eNOS) activation and increased agonist-induced phosphorylation of the EDG-1 receptor (Igarashi and Michel 2000). Thus, in conclusion, these studies have shown that the receptors can be located to caveolae, and that Cav1 can be positive, negative or have no impact on the downstream signaling.
In Cav1(-/-) brown pre-adipocyte cultures, the ablation of Cav1 did not influence the LPA-induced Erk1/2 activation on a short-term basis (paper IV). This is thus in contrast to the Erk1/2 activation findings of Kranenburg et al. (Kranenburg et al. 2001). As Gobeil et al. found that the LPA Rs are in caveolae but the signaling to Cox-2 mRNA expression is not affected by cholesterol-disturbing agents (Gobeil et al. 2003), it is possible that the LPA receptors in brown pre-adipocytes can be localized to caveolae even though the signaling pathway to Erk1/2 are not dependent on Cav1.

5.5 EGF receptors

The epidermal growth factor receptors (EGFRs) are receptor tyrosine kinases (RTKs) and belong to the ErbB family. In the following section, the ErbB family of receptors and their ligands and signaling will be discussed, but the focus will lie on the EGFRs, especially regarding the interaction with the caveola/caveolin (see below). The ErbB family consists of four members: EGFR (/ErbB1/human EGF receptor 1(HER1)), ErbB2 (/Neu/HER2), ErbB3 (/HER3) and ErbB4 (/HER4). All receptors have an extracellular ligand-binding domain, a cytoplasmic protein tyrosine kinase domain, and a membrane-spanning domain (Olayioye et al. 2000, Hynes et al. 2001, Prenzel et al. 2001). Many dimerization patterns exist, both as homo- and heterodimers (e.g. ErbB1/ErbB1, ErbB1/ErbB2, ErbB2/ErbB3, ErbB4/ErbB4 and ErbB2/ErbB4), although the ErbB3/ErbB3 cannot signal due to impaired kinase activity (Olayioye et al. 2000, Jorissen et al. 2003). ErbB2 seems to be the preferred heterodimerization partner for the other family members, and one function of ErbB2 is to decrease ligand dissociation from the receptor complex (Olayioye et al. 2000, Prenzel et al. 2001). The ErbB receptors are expressed in several tissues with an epithelial, mesenchymal and neuronal origin. ErbB receptors have been implicated in development, proliferation, and differentiation. Also deregulation of ErbB expression seems to play an important role in development and malignancy of numerous types of cancers (Olayioye et al. 2000, Holbro et al. 2003). The EGFR ligand EGF has been shown to stimulate the proliferation and inhibit the differentiation of rat adipocyte precursors isolated from inguinal fat pads (Serrero 1987). Regarding brown adipose tissue, EGF has been shown to induce proliferation in cultures of rat fetal brown adipocyte cultures (Valverde et al. 1991), in rat brown adipocytes (Garcia and Obregon 1997, Garcia and Obregon 2002), in a brown adipocyte-like cell line (Nakano et al. 2007), and in mouse brown pre-adipocytes (paper II). In addition, EGF inhibits uncoupling protein 1 (UCP1) gene expression in rat brown adipocytes, thus inhibiting differentiation (Garcia and Obregon 2002).
ErbBx-ablated mice have been investigated and as reviewed in Olayioye et al. (Olayioye et al. 2000), ErbB1-ablation mice leads to embryonic or perinatal lethality (depending on the genetic background), and the mice have abnormalities in multiple organs such as brain, skin, lung and gastrointestinal tract (generated by (Miettinen et al. 1995), (Sibilia and Wagner. 1995), and (Threadgill et al. 1995)). The ErbB2-ablated mice and ErbB4-ablated mice die at midgestation (E10.5) due to malformations in the heart (ErbB2+/− mice generated by (Lee et al. 1995) and (Erickson et al. 1997), and ErbB4+/− generated by (Gassmann et al. 1995)). The ErbB3-ablated mice die around E13.5 and have defective valve formation in the heart (generated by (Riethammer et al. 1997) and (Erickson et al. 1997)).

The ligands that bind to the ErbB receptors are a family of ligands – the EGF-related peptide growth factors. They can be classified into 4 subgroups depending on their binding: 1) EGF, amphiregulin and transforming growth factor-α (TGF-α) can bind ErbB1, 2) betacellulin, heparin-binding EGF (HB-EGF) and epiregulin can bind both ErbB1 and ErbB4, 3) neuregulins (NRG)-1 and -2 can bind ErbB3 and ErbB4, and 4) NRG-3 and -4 can bind ErbB4. For ErbB2 no direct ligand has been found, and ErbB2 is now thought to function as a coreceptor (see Fig. 14) (Olayioye et al. 2000, Hynes et al. 2001, Prenzel et al. 2001, Harris et al. 2003). The ligands are generally synthesized as glycosylated transmembrane precursors that are proteolytically cleaved from the cell surface; however, they can also be active before this cleavage (Massague and Pandiella 1993, Prenzel et al. 2001). Most of the ErbB ligands will act in an autocrine or a paracrine fashion, and the availability of the ligand will decide whether a specific signaling pathway is activated or not (Olayioye et al. 2000, Harris et al. 2003). The ligands are bivalent and are able to influence which receptor dimer that is formed and thereby which signaling that should take place. The ligands also possess different binding affinities (Olayioye et al. 2000, Hynes et al. 2001). There have been many studies investigating how the ligands bind to the receptor; however, this will not be discussed here (see cited reviews (Heldin 1995, Lemmon et al. 1997, Olayioye et al. 2000, Jorissen et al. 2003).

Not only the above-mentioned ErbB ligands can activate the ErbB receptors; a process named transactivation can occur (as discussed above for LPA signaling). Transactivation is the activation of e.g. MAP kinases (leading to gene transcription and proliferation (Hynes et al. 2001, Prenzel et al. 2001)) by an EGFR that has been activated indirectly from an activated GPCR (such as the LPAR). For more reading about transactivation, see (Prenzel et al. 1999, Hynes et al. 2001, Prenzel et al. 2001, Pai et al. 2002, Holbro et al. 2003, Harris et al. 2003, Gschwind et al. 2003, Madarame et al. 2003).
The ErbB receptors are constantly cycled between the plasma membrane and the endosomal compartment. They are internalized (half-time about 30 min) but then quickly recycled. This process is somewhat slower for the EGFRs than the rest of the ErbB receptors. However, upon ligand binding, the EGFRs have accelerated internalization and increased lysosomal targeting, and compared to the other members, EGFR signaling occurs mostly from the endosomes (Baulida et al. 1996, Prenzel et al. 2001, Wiley 2003). The internalization can take place via clathrin-coated pits, caveolae, and circular dorsal ruffles (Beguinot et al. 1984, Baulida et al. 1996, Prenzel et al. 2001, Puri et al. 2005, Sigismund et al. 2005, Orth and McNiven 2006). The internalization has been shown to involve monoubiquitination with the ubiquitin ligase Cbl and the adaptor molecule CIN85 (Cbl-interacting protein of 85 kDa) (Dikic 2003, Haglund et al. 2003a, Haglund et al. 2003b). Ligand-dependent mono-ubiquitination and subsequent receptor internalization and degradation have also been shown for the PDGFRs (Haglund et al. 2003b). Sprouty2, a protein that can interact with Cbl and CIN85, has been shown to inhibit downregulation of EGFR (Haglund et al. 2005).

**Mediators of signaling**

Upon ligand binding, ErbB will homo- or hetero-dimerize. This will activate intrinsic tyrosine kinase activity and lead to autophosphorylation of specific tyrosine residues in the intracellular domain. These phosphorylations will act as docking sites for signaling molecules as discussed below. Depending on the ligand that is bound and what dimerization took place, this will lead to different effects in the cell, e.g. gene expression and other biological responses (Olayioye et al. 2000, Hynes et al. 2001, Prenzel et al. 2001, Jorissen et al. 2003).

The signaling mediators of ErbB signaling are usually proteins containing Src homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains.
No general signaling pathway has been found between the cell types investigated, but some of the candidates are the adaptor proteins Shc, Crk, Grb2, Grb7 and Gab1, and kinases like Src, PI3K and Chk. Also, the protein tyrosine phosphatases SHP1 and SHP2, ras-GAP, PLD, PLCγ, c-Abl, c-Cbl, Eps15, JAK/STAT, p38 and β-arrestin have been implicated (Hu et al. 1992, Olayioye et al. 2000, Hynes et al. 2001, Prenzel et al. 2001, Jorissen et al. 2003, Frey et al. 2006, Hupfeld and Olefsky 2007).

In many cell types, EGF controls cell proliferation through the Erk1/2 MAP kinase pathway. One pathway to activate Erk1/2 is via Shc and Grb2 (growth factor receptor bound 2)/Sos (son of sevenless, a GEF (guanine-nucleotide exchange factor). Sos will recruit the G-protein Ras that can then activate the serine-threonine kinase Raf. Raf in turn activates MEK1 that then phosphorylates Erk1/2. Downstream of Erk1/2 are e.g. the transcription factors Elk-1 and c-fos that can regulate gene expression (Buday and Downward 1993, Yart et al. 2001, Prenzel et al. 2001, Jorissen et al. 2003,). However, also PI3K/Akt (Wennstrom and Downward. 1999, Yart et al. 2001, Chaudhary and Hruska. 2001), PKC (Corbit et al. 2000, Cohen et al. 2006) and Src (Roche et al. 1995, Olayioye et al. 2001, Krymskaya et al. 2005) can mediate Erk1/2 activation or cell proliferation upon EGF stimulation.

In brown pre-adipocytes, we found that Erk1/2 is a common and essential step in EGF-stimulated cell proliferation (see paper II). The EGF-induced Erk1/2 activation was only partially dependent on Src, and another pathway to the MAP/Erk kinase (MEK) exists. EGF also induced the PI3K-Akt pathway; however, this did not lead to Erk1/2 activation. Additionally, PKCs were not involved in EGF-induced Erk1/2 activation. In Fig. 15, the EGF signaling pathway in brown adipose tissue is depicted.

**Interaction with caveolin and caveolae**

Of the main receptors discussed in this thesis, the ErbB receptors and the influence of caveolae/caveolin on their localization and signaling have been by far the most investigated. Perhaps one reason is the implication of both the ErbB receptors and caveolin-1 in cancer (Cav1 may act as a tumor suppressor (compare section 2.2)). In this section, I have limited the content to mainly focus on the influence of Cav on EGFR localization and the signaling pathways leading to Erk1/2 activation.

The EGFRs have been shown to both colocalize and not to colocalize with caveolin/caveolae (see reviews by (Pike 2005, de Laurentiis et al. 2007)). EGFR has been cofractionated with the caveolae/lipid raft fractions in normal human fibroblasts (Smart et al. 1995), in A431 cells (Couet et al. 1997) and in PAC-1 smooth muscle cells (rat pulmonary artery) (Liu et al. 2007). Also in Rat1 cells, normal human fibroblasts (Mineo et al. 1996, Mineo et al. 1999), human bronchial smooth muscle cell line (Gosens et al. 2007) and rat liver (Wang et al. 2009) EGFR colocalized with the caveo-
lae/lipid raft fractions. However, upon ligand binding in these cells the EGFR was reduced from the caveolae/lipid raft fraction either by moving to the bulk membrane and/or being endocytosed.

![Diagram of EGF and PDGF signaling in brown adipose tissue](image)

**Fig. 15.** EGF and PDGF signaling in brown adipose tissue.

That the EGFR was able to colocalize with Cav1 was shown by Couet et al. by co-immunoprecipitation (Couet et al. 1997). However, in the same cell line, in a study by Waugh et al., EGFR did not co-immunoprecipitate with Cav1, and the conclusion was that the EGFR resided in lipid rafts (Waugh et al. 1999), thus the opposite of the study by Couet et al. In addition, in contrast to the study in rat liver cells above by Wang et al., Pol et al. did not find any EGFR in the caveolae-enriched plasma membrane fraction in rat liver (Pol et al. 1998). Furthermore, Matveev and Smart found that in Swiss 3T3 cells, EGFR cofractionated with Cav1 but they did not co-immunoprecipitate; however, upon ligand binding, the EGFR was relocalized and now co-immunoprecipitated with Cav1 (Matveev and Smart 2002).

Upon ligand binding, the EGFR is thought to be endocytosed. Matveev and Smart found that in Cav1-deficient Swiss 3T3 cells, EGFR was not internalized upon ligand binding; thus EGFR internalization was dependent on Cav1 (Matveev and Smart 2002). In contrast, Kazazic et al. found in HeLa...
and HEp2 cells that EGFR was internalized via clathrin-coated pits and not by caveolae/lipid rafts upon ligand binding (the EGFR was also not colocalized to caveolae) (Kazazic et al. 2006). In agreement, Khan et al. found the same results with EGFR in A549 human lung carcinoma cells. However, under oxidative stress in these cells the EGFR was colocalized with Cav1 at the plasma membrane, before being sorted to a perinuclear compartment via a clathrin-independent, caveolae-mediated pathway. This also led to the EGFR not being degraded (Khan et al. 2006). Additionally, Sigismund et al. found that EGFRs were internalized via clathrin-coated pits upon stimulation with low EGF concentrations, but at higher concentrations of EGF, a fraction of the EGFRs were ubiquitinated and this led to internalization via a caveolae/lipid raft-dependent pathway (Sigismund et al. 2005). Thus, in some cell systems and depending on activation, the EGFR is internalized via either caveolae or other endocytotic pathways, such as clathrin-mediated pathways.

The localization of EGFR in endosomes has also been investigated. In rat liver endosomes, EGFR was cofractionated with Cav1 in the early-sorting endosomes and the recycling receptor compartment (Pol et al. 1998). Balbis et al. also found that upon EGF stimulation, the EGFR was internalized into endosomes and the EGFR was found both in detergent-resistant membranes (DRM) and the bulk membrane (Cav and flotillin-1 was only present in DRM). However, no co-immunoprecipitation with Cav1 could be found (Balbis et al. 2007) and it seems that the EGFR is not in the Cav1-DRM parts of the endosomes and instead in a lipid raft-part. Thus, in conclusion, the EGFR can reside both in the caveolae/lipid raft fraction and in the bulk membrane of the endosomes. The p38 MAPK seems to play a part in the endocytotic process and in YAMC cells (young adult mouse colon), treatment with a p38 MAPK inhibitor caused the EGFR to accumulate in the endosomes and colocalize with Cav1 (Frey et al. 2006).

From the studies above, it is hard to say whether the EGFR is present or not in caveolae. Most studies have used fractionation and it is not possible to distinguish between caveolae and lipid rafts. Also in the study by Wang et al., they utilized three different methods to isolate caveolae/rafts – one dependent on detergent (according to (Liu and Anderson 1995)) and two not dependent on detergents (optiprep as (Smart et al. 1995) and Na2CO3 as (Song et al. 1996)). Dependent on the method used, they found different results; e.g. the two non-detergent methods showed an immediate decrease in EGFR after stimulation with EGF (2 min) in the caveolae/lipid rafts (as did the plasma membrane), while the detergent method showed an increase in receptors in caveolae/lipid rafts at this time point. In addition, only with this method did the caveolae/lipid raft fraction contain phosphorylated EGFR (Wang et al. 2009). Thus, the method used to isolate caveolae/lipid rafts seems to influence the result (see discussion in section 2.4).

By using immunoprecipitation, a direct interaction with Cav1 can be tested, and Couet et al. found that the EGFR could interact with the caveolin-
scaffolding domain (CSD). It is thought that the domain stabilizes the EGFR in an inactive conformation (Couet et al. 1997, de Laurentiis et al. 2007). However, since Waugh et al. found the opposite in the same cell line (Waugh et al. 1999), it is hard to draw any conclusion based on immunoprecipitation. By confocal microscopy, Roepstorff et al. found that the EGFR colocalized with GM1 and not Cav1 (Roepstorff et al. 2002), and by immunoelectron microscopy, Ringerike et al. found that EGFR did not localize to caveolae (Ringerike et al. 2002). Thus, these two studies point to the fact that the EGFR is not localized to caveolae but to lipid rafts.

In summary, by taking all of these studies together, these scenarios might take place: 1) EGFR is present in caveolae, and upon ligand binding moves outside; 2) EGFR is not present in caveolae, but upon ligand binding moves to caveolae or 3) EGFR is not present in caveolae, and upon ligand binding does not move to caveolae (here I have distinguished between caveolae and lipid rafts).

Binding of EGF to the receptor has been shown to be increased when the cells are cholesterol-depleted with methyl-β-cyclodextrin (mβCD) (Roepstorff et al. 2002, Ringerike et al. 2002, Pike and Casey 2002). In addition, the agent U18666A (causes a redistribution of cholesterol from the plasma membrane to late endosomes) increased EGF binding (Ringerike et al. 2002). However, filipin (causes aggregation of cholesterol via crosslinking) did not change the EGF binding (Roepstorff et al. 2002). By adding cholesterol to the cells, EGF binding was reduced (Roepstorff et al. 2002, Ringerike et al. 2002), which is consistent with the use of cholesterol-disturbing agents.

Treatment with both mβCD and EGF induced more EGFR dimerization than with only EGF, and there were more EGFRs at the plasma membrane. Also, with the addition of water-soluble cholesterol analogues and EGF, the EGFRs did not dimerize and there were less receptors at the plasma membrane (Ringerike et al. 2002).

The phosphorylation of the receptor is increased when cells are treated with mβCD (Ringerike et al. 2002, Westover et al. 2003, Liu et al. 2007). However, mβCD has also been found not to influence the phosphorylation (Jang et al. 2001, Pike and Casey 2002); this difference was not dependent on cell type since Ringerike et al., Westover et al. and Jang et al. used the same cell line (A431), nor dependent on the concentration of mβCD. The mβCD has also an effect on the EGF-induced EGFR phosphorylation, causing an increase in phosphorylation (Ringerike et al. 2002, Westover et al. 2003) and mβCD also increased the intrinsic kinase activity of the receptors (Pike and Casey 2002, Westover et al. 2003).

Thus, in summary, cholesterol and caveolae/lipid rafts play a role in the EGF binding ability, and the dimerization and phosphorylation of the EGFRs.
The importance of caveolae/lipid rafts for EGF-induced Erk1/2 activation has been investigated. mβCD can by itself increase Erk1/2 phosphorylation (Furuchi and Anderson 1998, Chen and Resh 2001, Liu et al. 2007), but has also been shown not to affect Erk1/2 phosphorylation levels (Park and Han 2009). Also, Yin et al. found that neither filipin nor nystatin treatment affected the basal Erk1/2 phosphorylation levels, and neither did transfection with Cav1 siRNA (Yin et al. 2008).

Upon treatment with mβCD and EGF stimulation in Rat-1 and NIH-3T3 cells, Erk1/2 phosphorylation levels are augmented (Furuchi and Anderson 1998, Pike and Casey 2002). In contrast, Park et al. found that both mβCD and Cav1 siRNA could inhibit EGF-induced Erk1/2 activation in mouse embryonic stem (ES) cells (Park and Han 2009), and also Kranenburg et al. found that treatment with mβCD in Cos-7 cells inhibited the EGF-induced MEK/Erk1/2 activation (Kranenburg et al. 2001). However, in PC12 cells, treatment with mβCD or filipin did not affect EGF-induced Erk1/2 phosphorylation (Peiro et al. 2000). Additionally, in hepatic C9 cells, treatment with nystatin, filipin or transfection with Cav1 siRNA upon EGF-stimulation did not affect the Erk1/2 phosphorylation levels and neither EGFR nor Src phosphorylation levels were changed; however, Akt phosphorylation was inhibited by both nystatin and filipin, but not by Cav1 siRNA (Yin et al. 2008). Thus, in these two studies, Cav1 and cholesterol were not important for EGF-induced Erk1/2 signaling.

Furthermore, the effect of age on Erk signaling has also been investigated. In young human diploid fibroblasts but not in old, EGF is able to induce Erk1/2 phosphorylation. Park et al. found that the old cells had an increase in all Cav subtypes and that EGFR was immunoprecipitated with Cav, while young cells had lower Cav expression and EGFR did not coimmunoprecipitate. By overexpressing Cav1 in young cells, Erk1/2 could no longer be phosphorylated. Also in tissues from old rats (e.g. brain, spleen, lung) Cav expression was upregulated (Park et al. 2000). Thus, with increasing age, Cav1 is upregulated and can then inhibit EGF-induced Erk1/2 signaling.

In brown adipose tissue, we found that EGF-induced Erk1/2 phosphorylation was not changed in Cav1(-/-) brown pre-adipocytes (see paper IV). EGF was able to stimulate Erk1/2 in the same concentration-response manner as in the wild-type cultures. Thus, Cav1 seems not to be of importance for this short-term signaling. On a long-term basis, there seems to be no difference in the cell proliferation, since no difference in the amount of total Erk1/2 could be found. However, whether the EGFR resides in the caveolae and whether the EGFR is endocytosed by caveolae remains to be investigated.

In summary, it seems that depending on cell type, caveolae/lipid rafts might play different roles in the regulation of EGF-induced Erk1/2 activation, with it sometimes being inhibitory, sometimes stimulatory and sometimes making no difference.
In contrast to the EGF results in the hepatic C9 cells used by Yin et al., nystatin, filipin and Cav1 siRNA inhibited angiotensin II (AngII)-induced phosphorylation of Erk1/2 (Yin et al. 2008). The AngII-induced Erk1/2 activation is predominantly thought to occur via transactivation of EGFR (Shah 2002) and also the phosphorylation of EGFR, Src, Akt and Cav1 was inhibited (Yin et al. 2008). Thus, for AngII-induced Erk1/2 signaling, Cav1 and cholesterol were of importance. For further reading about caveolae/lipid rafts in AngII-induced Erk1/2 activation see (Ushio-Fukai et al. 2001, Shah. 2002, Olivares-Reyes et al. 2005). Caveolae/lipid rafts have also been implicated in endothelin-1/EGFR transactivation/Erk1/2-signaling (Hua et al. 2003) and in oxytocin receptor/EGFR transactivation/Erk1/2-signaling (Rimoldi et al. 2003).

AngII stimulation increased Cav1-phosphorylation. In addition, stimulation with EGF leads to phosphorylation of Cav1 (Lee et al. 2000, Gosens et al. 2007, Yin et al. 2008, Park and Han 2009). Yin et al. showed that the EGF-induced Cav1 phosphorylation was dependent on Src, EGFR, Ca\(^{2+}\), but not on PI3K (Yin et al. 2008). Additionally, Kim et al. showed that both C-terminal-truncated EGFR and overexpressed wild-type EGFR were able to phosphorylate Cav1 more than the regular wild-type EGFR or a kinase-inactive EGFR. Both truncation and overexpression of the EGFR have been linked to cell transformations (Kim et al. 2000).

Stimulation of Erk1/2 by EGF can induce cell proliferation. In Rat-1 cells, cell proliferation was increased by mβCD itself and augmented further when cells were treated with EGF (Furuchi and Anderson 1998). In agreement, Park et al. found that in mouse ES cells, both mβCD and Cav1 siRNA inhibited EGF-induced cell proliferation. In these cells, the Cav1 siRNA also inhibited EGF-induced cell migration, EGF-induced expression of MMP2, the proto-oncogenes c-fos, c-myc, c-jun, and the cell cycle regulatory proteins cyclin D1, CDK4, cyclin E and CDK2 (Park and Han 2009).

The effect on Elk activation has also been investigated. In CHO cells overexpressing EGFR, Erk1/2 activates Elk. When these cells co-expressed Cav1, Elk activation was inhibited. The inhibitory part of Cav1 was found to be within the residues 32-95. In addition, overexpression of Cav1 inhibited the Raf/MEK/Erk-mediated signaling to Elk (Engelman et al. 1998).

Other mediators in the EGF-induced Erk1/2 signaling pathway have been investigated concerning Cav1 and cholesterol. Shc, Grb2, \(G_{\alpha_i}\), \(G_{\beta}\), K-Ras, H-Ras, N-Ras and Src have been found to be located to the caveolae/lipid raft fraction (Furuchi and Anderson 1998, Kranenburg et al. 2001, Wang et al. 2009). Furuchi et al found that treatment with mβCD reduced the amount of Cav1, Grb2, K-Ras, H-Ras, Src, Shc, mG\(_{\beta}\) and Erk1/2 and that there was no affect on \(G_{\alpha_i}\) in these fractions. In addition, EGF stimulation of mβCD-
treated cells reduced the level of Sos, c-Raf and Shc compared to only EGF stimulation (Furuchi and Anderson 1998). Kranenburg et al. found that there was a difference between EGF-stimulated K-Ras and N-Ras signaling. EGF could only activate N-Ras, that was located both in caveolae/lipid rafts and outside, and not K-Ras, that was located only in the caveolae/lipid raft fraction. When the cells were treated with mβCD, EGF could activate both Ras isoforms, and EGF-induced MEK/Erk1/2 were inhibited (Kranenburg et al. 2001). In contrast to the studies above, Gosens et al. could not find Grb2, Raf-1, or Erk1/2 in the caveolae/lipid raft fraction, and there was no difference in localization upon EGF stimulation (Gosens et al. 2007).

PLCγ and PLD have been investigated in regard to EGF signaling and caveolae/lipid rafts. Jang et al. found that only a small amount of PLCγ was found in the Cav1 fraction, but upon EGF stimulation it was translocated to the caveolae fraction. mβCD inhibited the translocation and also inhibited the formation of inositol phosphates (Jang et al. 2001). Han et al. found that EGF-induced phosphorylation and activation of PLD occurred in the caveolae-enriched membrane fraction and that the correct localization of PLD to these parts is critical for EGF signaling (Han et al. 2002).

In conclusion, Cav1 and cholesterol-disturbing agents can affect the EGF-induced signaling concerning EGF binding, EGFR dimerization and autophosphorylation, Erk1/2 activation and cellular functions such as proliferation. Whether the differences seen between some cell types and others are dependent on cell type or simply on methodology, will hopefully be further addressed when better easy-to-use methods to distinguish between caveolae and lipid rafts are engineered.

5.6 PDGF receptors

Similarly to EGFRs, the platelet-derived growth factor receptors (PDGFRs) belong to the receptor tyrosine kinases (RTKs). The PDGFRs can be of either an α- or β-receptor isoform. The receptors can both homodimerize (αα or ββ) or heterodimerize (αβ) upon ligand binding. Both forms are structurally similar with five immunoglobulin-like domains on the extracellular domain, and a split kinase domain with one intervening sequence in the intracellular domain (Heldin et al. 1998, Heldin and Westermark 1999, Heldin 2004, de Laurentiis et al. 2007). PDGFR are expressed in a wide range of cell types, such as fibroblasts, myoblasts, vascular smooth muscle cells, macrophages, platelets and 3T3-L1, just to mention a few (Heldin and Westermark 1999, Shigematsu et al. 2001).

The agonist PDGF consists of dimers of structurally similar polypeptide chains bound together by sulfide bonds. There are four different polypeptide
chains, A, B, C and D, where the A exists in two different splice variants. The chains can either homodimerize as AA, BB, CC or DD or heterodimerize as AB. The chains can be divided in one subfamily with A and B and one with C and D. This is based on the fact that A and B are secreted in their active form (the N-terminal prodromains are cleaved intracellularly), while C and D are activated after secretion (by cleavage of the N-terminal CUB domain (CUB = complement subcomponents C1r/C1s, sea urchin uEGF and human BMP-1) (Hoch and Soriano 2003). The different ligands have different receptor specificity with the A, B, and C being able to bind to \( \alpha \)-receptors, and B and D being able to bind to \( \beta \)-receptors. Thus, the PDGF-AA binds only to PDGFR\( \alpha \alpha \), PDGF-BB bind to all PDGF receptor types, PDGF-DD to PDGFR\( \alpha \beta \) and \( \beta \beta \), and PDGF-CC and -AB to PDGFR\( \alpha \alpha \) and \( \alpha \beta \) (however, according to Heldin et al., the PDGF-CC only binds to PDGFR\( \alpha \alpha \) (Heldin 2004)) (see Fig. 16). Due to the different binding specificity, different ligands interacting with different receptors will give different cellular responses (Heldin et al. 1998, Hoch and Soriano 2003, Heldin 2004).

Not only PDGFs can activate the receptors; also, transactivation by G-protein coupled receptors is a possible mechanism (Herrlich et al. 1998, Goppelt-Struebe et al. 2000) as also discussed in the LPA and EGFR sections.

Fig. 16. PDGF ligands and receptors. Modified from (Hoch and Soriano 2003, Heldin 2004).

PDGF signaling is involved in cell growth (initiation of DNA synthesis and cell division), cell differentiation, anti-apoptosis, cell transformation, and migration. It also promotes wound healing, stimulates chemotaxis, and growth of many cells involved in healing processes. PDGF signaling has been implicated in diseases, e.g. atherosclerosis, fibrosis, and malignant diseases (Heldin et al. 1998, Hoch and Soriano 2003, Heldin 2004). Different mice ablated of certain parts of the ligand or the receptors have been derived to elucidate the function of PDGFR signaling. For example, PDGF-B chain- or PDGFR\( \beta \)-ablated mice have severe defects in the development of the
kidneys and blood vessels (the vascular smooth muscle cells and the pericytes; death occurs at time of birth) (PDGF-B−/− generated by (Leveen et al. 1994) and PDGFβ−/− generated by (Soriano 1994)). Furthermore, ablation of the PDGF-A chain leads to the death of these mice and they have defective development of the alveolar smooth muscle cell progenitors (generated by (Bostrom et al. 1996)), and PDGFRα-ablated mice have a severe phenotype with cranial malformations and deficiency in myotome formation (generated by (Soriano 1997)) (Heldin and Westermark 1999).

Upon ligand binding, the α-receptors and/or the β-receptors will dimerize. This will cause autophosphorylation of the receptors. Phosphorylation in the kinase domain of the receptor will enhance the kinase activity of the receptors (this binding site is conserved between the receptor forms (β-Tyr857 and α-Tyr849 in the kinase domain)), and phosphorylation of tyrosine residues outside the kinase domain will create docking sites for signaling molecules (discussed below) (Heldin et al. 1998, Hoch and Soriano 2003, Heldin 2004).

Mediators of signaling

Mainly Src homology 2 (SH2)-domain containing signaling molecules binds to the activated receptors. However, also molecules with SH3 domains, phosphotyrosine binding (PTB) domains, pleckstrin homology (PH) domains, and PDZ domains have been implicated. Typical SH2-domain containing mediators are PI3K, PLCγ, Grb2, Src, Stat (signal transducers and activators of transcription), GAP (GTPase activating protein), and Shc (for review see Heldin et al. 1998 and 1999) (Heldin et al. 1998, Heldin and Westermark 1999, Heldin 2004, de Laurentiis et al. 2007). In addition, GRK2 and β-arrestins have been implicated in PDGF signaling (Hupfeld and Olefsky 2007).

PI3K has been implicated in PDGF signaling for inducing cell growth, survival, and motility (Heldin et al. 1998, Heldin. 2004). PI3K has been shown to bind to activated PDGFRs (Coughlin et al. 1989, Yu et al. 1994). Further downstream, PI3K is able to activate e.g. Akt/protein kinase B (PKB), protein kinase C (PKC), p70S6K, Rho family of GTPases, and Erk1/2 (Heldin et al. 1998, Taylor 2000, Chaudhary and Hruska 2001, Tsakiridis et al. 2001, Osaki et al. 2004, Hennessy et al. 2005).

PLCγ can activate IP3 formation, which leads to an increase in intracellular Ca2+ levels. This in turn can activate classical PKCs via a phospholipase D (PLD)-diacylglycerol (DAG) pathway. PLCγ has been implicated in the motility effects of PDGFR signaling (Heldin et al. 1998). The PKCs have also been implicated in PDGF signaling; Kobayashi et al. found that in rat hepatic stellate cells, PDGF was able to phosphorylate myristoylated alanine-rich C-kinase substrate (MARCKS), a specific substrate of PKC. Additionally, PDGF was able to phosphorylate Erk1/2, PKB, and p70S6 kinase (Kobayashi et al. 2007).
The growth factor receptor bound 2 (Grb2) adaptor molecule interacts with Sos1 (son of sevenless) to activate Ras through nucleotide exchange. Ras can then activate Raf-1 (a mitogen-activated protein kinase kinase kinase (MAPK/Erk kinase kinase)), which in turn activates MAPKK or MEK, which in turn activates Erk1/2. This pathway has been implicated in the mitogenic effects of PDGF. In this pathway, there is negative feedback since Sos1 can be inhibited by MAP kinases, which leads to decreased Ras signaling (Heldin et al. 1998, Heldin 2004).

Src tyrosine kinases often signal from receptor tyrosine kinases that can lead to Erk1/2 activation and proliferation (Roche et al. 1995, Heldin et al. 1998, Krymskaya et al. 2005, Ali et al. 2005, Chaturvedi and Sarkar 2005), and it is thought that the mitogenic effects of PDGF are partially dependent on Src (Heldin 2004).

As was the case for EGFR, ligand-dependent mono-ubiquitination and subsequent receptor internalization and degradation have also been shown for the PDGFRs (Haglund et al. 2003b).

PDGF has previously been shown to induce proliferation in cultures of rat brown adipocytes (Garcia and Obregon 1997). In paper II, we found that in mouse brown pre-adipocytes, Erk1/2 is a common and essential step in PDGF-stimulated cell proliferation. In comparison to the EGF signaling pathway to Erk1/2 activation described above, the PDGF-induced Erk1/2 activation pathway was via PI3K, PKC (possibly ζ), and Src. PI3K was able to activate Akt/PKB; however, Akt/PKB was not involved in the signaling to Erk1/2 MAP kinase (paper II). In Fig. 15, the PDGF signaling pathway in brown adipose tissue is depicted.

Interaction with caveolin and caveolae

The interactions of the PDGFR with caveolin/caveolae have been moderately studied. The PDGFRs have been shown to both colocalize and not to colocalize with caveolin (see reviews (Pike 2005, de Laurentiis et al. 2007)). The receptors were cofractionated with Cav1 in normal human fibroblasts (Liu et al. 1996, Liu et al. 1997b), rat lung microvascular endothelial cells (Liu et al. 1997a), NIH-3T3 fibroblasts (Yamamoto et al. 1999), Swiss 3T3 cells (Matveev and Smart. 2002, Mitsuda et al. 2002), and airway smooth muscle cells (Gosens et al. 2006) indicating that the PDGFR localized to caveolae/lipid rafts. The method used by Liu et al. (Liu et al. 1997a) was designed to isolate caveolae from lipid rafts, and the PDGFR was in the caveolae fraction. That PDGFR can colocalize with Cav1 was shown by immunoelectron microscopy (Liu et al. 1996, Liu et al. 1997b) and by immunoprecipitation (Yamamoto et al. 1999, Gosens et al. 2006). However, Matveev and Smart found that the PDGFR co-immunoprecipitated with CD55, a lipid raft marker, and not with Cav1 (Matveev and Smart 2002). Additionally, soluble PDGF-BB activates a cohort of PDGFRβ that are located to caveolae/lipid rafts, while the cell-bound PDGF-BB (cell-to-cell contact) acti-
vated PDGFRβ that are located to the bulk membrane in the same cells (Sundberg et al. 2009). Thus, in summary, these studies point to the receptors being located to different compartments, being activated differently depending on ligand and resulting in different signaling pathways and endpoints.

Upon stimulation with PDGF-BB, Yamamoto et al. found that the PDGFR remained in the same fractions as Cav1 (Yamamoto et al. 1999), while Liu et al. and Gosens et al. found that stimulation led to the dissociation of PDGFR from the Cav1 fraction (Liu et al. 1997, Gosens et al. 2006). Overexpressing the ganglioside GM1 in Swiss 3T3 cells also caused the PDGFR not to cofractionate with Cav1 (Mitsuda et al. 2002).

By using recombinant PDGFRs overexpressed in insect cells and different caveolin-x-peptides, Yamamoto et al. showed that Cav1 and Cav3 can bind PDGFR directly and that they can inhibit the autophosphorylation of PDGFR (Cav2 could not do this) (Yamamoto et al. 1999).

Phosphorylation of the PDGFR by PDGF has been shown not to be influenced by filipin and methyl-β-cyclodextrin (mβCD) treatment (Matveev and Smart 2002), and PDGF treatment has been shown to lead to phosphorylation of Cav1 in both airway smooth muscle cells (Gosens et al. 2006), human foreskin fibroblasts and 3T3-L1 cells (Newcomb and Mastick. 2002). However, Liu et al. found that treatment with filipin impaired the PDGF signaling (PDGF-induced tyrosine phosphorylation) (Liu et al. 1997a).

Regarding the signaling mediators, Liu et al. found that in normal human fibroblasts, p-PDGFR, PI3K, Src, Ras, Raf1, MEK, and Erk2 cofractionated with Cav (Liu et al. 1996, Liu et al. 1997b). Liu et al. found that in rat lung microvascular endothelial cells, PKC, PI3K, Src-like kinases (mainly Yes and Lyn), PLC, sphingomyelin, and phosphoinositides cofractionated with Cav (Liu et al. 1997a). Additionally, Michaely et al. found that in normal human fibroblasts, PDGF recruited RAC1 and RhoA to caveolae (Michaely et al. 1999). In primary cultures of smooth muscle cells, PDGF has been shown to recruit PP2A to a non-caveolin fraction, while it increased p34 in the caveolin fraction (Berrou and Bryckaert 2009).

Gosens et al. showed that in airway smooth muscle cells, treatment with Cav1 siRNA induced spontaneous Erk1/2 activation (also after methyl-β-cyclodextrin treatment) and increased cell proliferation. However, after PDGF stimulation of Cav1 siRNA-treated cells, the Erk1/2 phosphorylation was reduced. Thus, Cav1 is necessary for proper PDGF-induced Erk1/2 phosphorylation (Gosens et al. 2006). In contrast, Fujita et al. found that in Cav1-overexpressing rat primary mesengial cells, PDGF stimulation led to inhibition of Raf-1/MEK1/2/Erk1/2 activation and cell proliferation, and with a dominant negative caveolin mutant, the mesengial cells had an increased Erk1/2 activation. Thus, in these cells, Cav1 inhibits PDGF signaling to Erk1/2 (Fujita et al. 2004).
In brown adipocytes, we found that the PDGF-induced Erk1/2 phosphorylation was not changed in Cav1(-/-) brown pre-adipocytes (paper IV), thus in contrast to both Fujita et al. and Gosens et al., PDGF was able to stimulate Erk1/2 in the same concentration-response manner as in wild-type cultures. Similarly to the EGFR, Cav1 seems not to be of importance for short-term signaling. Whether the PDGFR resides in the caveolae and whether the PDGFR is endocytosed by caveolae remains to be investigated.

In conclusion, PDGFR seems to be located to both caveolae/lipid rafts and the bulk membrane. Depending on the location, different PDGF ligands might bind and transduce different signaling. For PDGF-induced Erk1/2 activation, Cav1 expression seems to be able to be both stimulatory and inhibitory or also not to affect the signaling at all. This difference could be due to cell-specific signaling.

5.7 Other receptors and mediators relevant for brown adipose tissue

5.7.1 Insulin receptor
The insulin receptors (InsRs) also belong to the receptor tyrosine kinases. They exist as disulphide-linked dimers. Each monomer is built up by one extracellular α- and one transmembrane β-chain. The ligands for the InsRs are insulin and the insulin-like growth factors (IGF), IGF-I, and IGF-II (Ward and Lawrence 2009). One of the functions in muscle and adipose tissue is the insulin-mediated translocation of glucose transporters (GLUTs, especially GLUT4) to the plasma membrane. In brown adipose tissue, the β-AR can mediate glucose uptake, as also briefly commented in section 5.1. The InsR has been shown to localize to caveolae, and the signaling is affected by caveolae/caveolin. However, the InsR will not be discussed in this thesis. The reader is directed to the work of Peter Strålfors’ group and the following reviews (Kandror and Pilch 1996, Cohen et al. 2003a, Saltiel and Pessin 2003, Ishikawa et al. 2005).

5.7.2 G-proteins and downstream signaling
In this section, I will briefly go through the view on G-proteins and their downstream signaling in relation to caveolin. For further reading, see (Insel et al. 2005, Anderson 2006, Willoughby and Cooper 2007, Patel et al. 2008b).
**G-proteins**

The heterotrimeric G-proteins are composed of a \( \alpha \)-, \( \beta \)- and \( \gamma \)-subunit. There are 15 different forms of the \( \alpha \)-subunit, 5 of the \( \beta \)-subunit and 12 of the \( \gamma \)-subunit. Based on the \( \alpha \)-subunit, the G-proteins can be divided into four families: \( G_{\text{i/o}} \), \( G_{q/11} \), \( G_{12/13} \), and \( G_{s} \). In the inactive form, G-proteins bind guanosine diphosphate (GDP), and in the active form guanosine triphosphate (GTP). Upon activation, the \( \alpha \)-subunit is dissociated from the \( \beta \gamma \)-subunits, and both the \( \alpha \)-subunit and the \( \beta \gamma \)-subunits can elicit signals to downstream effectors (Dupre et al. 2009).

G-proteins can be found in both caveolae/lipid rafts and in the bulk membrane. In addition, localization can differ between the caveolae and lipid rafts. Caveolin can interact with the \( \alpha \)-subunit, and binding to the caveolin-scaffolding domain is suggested to regulate the G-protein function (Patel et al. 2008b). For brown adipocyte signaling, the \( G_{q} \), \( G_{i} \) and \( G_{s} \) are important in coupling the \( \alpha_{1-} \), \( \alpha_{2-} \) (and LPA) and \( \beta \)-adrenergic signaling, respectively. \( G_{q} \), that stimulate increases in \( [Ca^{2+}]_{c} \), can be co-immunoprecipitated by Cav3 in rat cardiac tissue (Fujita et al. 2001) and the \( Ca^{2+} \) signaling has also been linked to caveolae/lipid rafts in different cell types (Pani and Singh 2009). \( G_{s} \) and \( G_{i} \), that stimulate or inhibit adenylyl cyclase respectively, will not be discussed any further here; the discussion will instead be on the adenylyl cyclase. For further reading about heterotrimeric G-proteins and caveolae, see e.g. (Patel et al. 2008a).

**Adenylyl cyclase and PKA**

The adenylyl cyclase (AC) family consists of one soluble and 9 membrane-bound isoforms. Some have been shown to be able to cofractionate and colocalize with caveolin (Willoughby and Cooper 2007, Patel et al. 2008b), and some of the AC can be inhibited by caveolin peptides (Toya et al. 1998). In addition, the receptor coupling efficiency to the ACs has been found to depend on the receptor number and the co-localization to caveolae (Ostrom et al. 2001). A majority of the work on AC and caveolin has been done in myocytes, and thereby the interaction with Cav3 was studied. For further reading about AC and its localization to caveolae, see e.g. (Insel et al. 2005, Pontier et al. 2008, Allen et al. 2009).

The function of AC is to convert ATP into cAMP. Since AC can be localized with caveolin, this can affect cAMP production. By depleting cells of cholesterol by methyl-\( \beta \)-cyclodextrin (m\( \beta \)CD), an increase in basal AC activity and an augmented forskolin-induced AC activity can be induced (Rybin et al. 2000, Pontier et al. 2008). However, m\( \beta \)CD can also inhibit forskolin-induced cAMP levels (Ostrom et al. 2004). Thus, caveolae/lipid rafts seem to play different roles in different cell systems.

Caveolin has been shown to affect cAMP levels directly. Toya et al. found that caveolin peptides could inhibit forskolin-induced cAMP levels.
(Toya et al. 1998). Additionally, Allen et al. found that forskolin-induced AC activity increased in the brains of Cav1-ablated mice compared to the wild-type mice, and that Cav1 siRNA could increase both forskolin-induced AC activity and cAMP levels in C6 glioma cells. Cav1 siRNA also increased isoprenaline-induced cAMP levels (Allen et al. 2009). Thus, from these studies, caveolin seems to inhibit AC signaling. This is in contrast to results in brown adipocytes (paper IV), where we see a decrease in forskolin-induced cAMP levels in Cav1(-/-) brown adipocytes compared to wild-type.

cAMP can be hydrolyzed by the phosphodiesteras (PDE) and thereby terminate the signaling. PDEs have been linked to caveolae/lipid rafts. For further reading, see (Nilsson et al. 2006, Patel et al. 2008b, Ahmad et al. 2009).

cAMP can activate protein kinase A (PKA). Caveolin can interact with PKA (Razani et al. 1999, Razani and Lisanti 2001), and the PKAc can colocalize with Cav1 (El-Yazbi et al. 2006). For further reading, see (Patel et al. 2008b).

Erk1/2 MAP kinase

Activation of extracellular-regulated protein kinase (Erk)1/2 is induced by many receptors, such as the adrenergic, LPA, EGF and PDGF receptors discussed above. Since Erk1/2 can be activated by many different ligands, the cells have a “control” system to regulate the different responses (Pouyssegur and Lenormand 2003, Ebisuya et al. 2005). Upon activation, Erk1/2 can be translocated to the nucleus and this can terminate the signaling (Brunet et al. 1999, Volmat et al. 2001).

Erk1/2 activation can be affected by both cholesterol and caveolin content. As discussed above, the adrenergic, LPA, EGF, and PDGF receptors can affect Erk1/2 activation to different extents upon disruption of cholesterol and caveolin. Here however, I will discuss the effect on Erk1/2 activation without agonist – e.g. the effect of cholesterol and caveolin/caveolae disruption. The cholesterol-depleting agent methyl-β-cyclodextrin can induce Erk1/2 activation (Furuchi and Anderson 1998, Chen and Resh 2001, Gosens et al. 2006, Liu et al. 2007), but has also been shown not to affect Erk1/2 activation (Park and Han 2009). In addition, the cholesterol-disturbing agents filipin and nystatin did not affect basal Erk1/2 phosphorylation levels (Yin et al. 2008). Thus, cholesterol can have both a positive and a negative influence on basal Erk1/2 activation. This is probably dependent on cell type.

Caveolin has been shown to inhibit both MEK-1 and Erk2 signaling (Engelman et al. 1998). Utilizing Cav1 siRNA, Gosens et al. found that Cav1 siRNA induced spontaneous Erk1/2 activation, while Yin et al. could not see a difference (Gosens et al. 2006, Yin et al. 2008). In Cav1-ablated mouse heart, Erk1/2 is hyperactivated compared to the wild-type mice (Cohen et al. 2003b); however, in Cav1(-/-) brown adipocytes no effect on basal Erk1/2
phosphorylation could be seen (compared to wild-type) (paper IV). Thus, the spontaneous activation of Erk1/2 by removal of caveolin seems to be a cell-type specific event.

For further reading about Erk1/2, see (Anderson 2006).

5.8 Conclusions of caveolin and signaling

The main receptors discussed in this section have all been implicated to signal via the caveolae and to interact with caveolins. Many studies have shown colocalization; however, several of the studies used fractionation, and with most fractionation techniques one cannot distinguish between caveolae and lipid rafts. Thus, even if the receptor cofractionated with caveolin, it does not necessarily mean that the receptor is located in caveolae, only that it is present in membrane rafts. It should also be noted that the localization to caveolae/lipid rafts is a dynamic process, with certain receptors and signaling mediators being transported in and out from these domains upon e.g. ligand stimulation. The localization to caveolae/lipid rafts is probably needed for certain signaling pathways, while for other it is inhibitory (see e.g. studies on the EGFR (section 5.5)).

Regarding signaling of the β3-adrenergic, LPA-, EGF, and PDGF-receptor to downstream targets, such as cAMP and Erk1/2, cholesterol-disturbing agents such as methyl-b-cyclodextrin, filipin, and nystatin have been used. However, these agents will not only affect caveolae, but also lipid rafts (and other components, see section 2.4) and no conclusion can be drawn about the influence of caveolae on signaling. In addition, siRNA against caveolin, and caveolin-ablated mice, have been used in signaling studies. Although these studies are more specific for caveolin, there are also problems with these methods. Since caveolins are not only located to caveolae, these studies more investigate the effect of caveolin and not caveolae; and since the caveolin-ablated mice are not conditional knockouts (i.e. the gene of interest has not been deleted in a tissue- or time-dependent manner), the effects seen in the mice could be due to secondary effects.

In general, investigations of the effect of caveolae (caveolin)/lipid rafts on signaling by the receptors discussed above have given inconclusive results. Whether this is an effect of methodology or cell type will hopefully be solved in the future.

In paper IV, we have used Cav1-ablated mice to investigate signaling in brown adipose tissue. We investigated the β3-adrenergic receptor (AR) signaling to cAMP (and adenylyl cyclase (AC) activity), and LPA-, EGF-, and PDGF-induced Erk1/2 activation. We found that only the β3-AR signaling to cAMP and AC activity was impaired by Cav1-ablation, and that LPA-, EGF- and PDGF-signaling to Erk1/2 was unaffected. In Fig. 17, a scheme of the
involvement of Cav1 on these signaling pathways is shown. Although the receptors have been placed either inside the caveolae or outside, the colocalization has not been investigated, and we cannot conclude anything about the localization. The figure should be regarded as showing that Cav1 only influences β3-AR-signaling to cAMP and AC activity, and not LPA-, EGF-, or PDGF-induced Erk1/2 activation.

Fig. 17. Scheme of involvement of Cav1 in β3-, LPA-, EGF-, PDGF-receptor and adenylyl cyclase signaling in brown adipose tissue. The receptors are located inside caveolae to show that Cav1-ablated had an effect on the signaling pathway, and the receptors are located outside if the Cav1-ablation did not have an effect on the signaling pathway.
Since the 1990’s, the plasma membrane has been considered to be heterogeneous with some parts being more loose and some more rigid. These rigid parts have been named membrane rafts. The membrane rafts consist of both lipid rafts and caveolae, which both contain a different lipid composition than the rest of the plasma membrane (Thomas and Smart 2008). The differences between lipid rafts and caveolae are that caveolae are invaginations in the plasma membrane (while lipid rafts are flat) and that caveolae contain the proteins caveolin (-1, -2 and -3) and cavin (-1, -2, -3 and -4) (which are required for the invagination). Caveolae have been implicated as being both signaling and metabolic platforms (Razani et al. 2002b, Ortegren et al. 2007) and seem to be able to regulate endocytosis, exocytosis, and cholesterol homeostasis (Thomas and Smart 2008).

Many methods can be used to investigate the effect of caveolae and caveolin in signaling pathways. However, some of these methods not only affect caveolae but also lipid rafts (e.g. most fractionation methods and use of cholesterol-disturbing agents), and if caveolin was found in the same fraction as the protein of interest, most studies would say that this protein colocalized with caveolae. This is a misconception, since lipid rafts would also be located in the same fraction, and one cannot distinguish between these domains with such techniques. The use of siRNA against Cav1 is more specific, but since Cav1 has been found outside caveolae, one could only conclude whether Cav1 has an effect, and not what role caveolae play. For colocalization studies, confocal microscopy and especially electron microscopy would be the best methods to use; however, this of course depends on the specificity of the antibodies used.

With the generation of Cav-ablated mice, more direct studies investigating the specific effects of caveolin are possible. However, one major caveat with these mice is that they are not conditional knockouts (i.e. the gene of interest has not been deleted in a tissue- or time-dependent manner). Thus the effects seen could be due to secondary effects; e.g. the lung phenotype in the Cav1-ablated mice seem to derive from the lowered expression of Cav2 (Le Lay and Kurzchalia 2005).

Cav1-ablated mice are resistant to diet-induced obesity and at older age Cav1-ablated mice are leaner than wild-type mice (Razani et al. 2002). Although differences have been found in serum metabolites between Cav1-
ablated and wild-type mice (Razani et al. 2002, Heimerl et al. 2008, Frank et al. 2008), and impairments in lipogenesis, lipid droplet formation and lipolysis have been seen (Cohen et al. 2004, Cohen et al. 2005, Le Lay et al. 2009, Blouin et al. 2009), the leanness and resistance to diet-induced obesity have not been explained. Further studies are needed to conclude the mechanism, and such studies should be performed at thermoneutrality (to reduce effects of increased metabolism).

On exposure to acute cold and fasting, Cav1-ablated mice cannot maintain their body temperature. On exposure to acute cold, mice rely on shivering for maintaining their body temperature (Golozoubova et al. 2004). To be able to shiver, the muscles require energy. This energy can come either from food intake or by lipolysis from white adipose tissue. Since Cav1-ablated mice have impaired lipolysis in white adipocytes (Cohen et al. 2004) and no food was given, Cav1-ablated mice had no substrate for shivering, and could therefore not maintain body temperature. To survive, Cav1-ablated mice allowed a regulated drop in body temperature (i.e. went into torpor). In Cav1-ablated mice only exposed to acute cold, this drop could not be seen, the probable cause being the supply of energy from food intake.

In prolonged cold, brown adipose tissue will become activated (with increased UCP1 expression) and shivering will be replaced by nonshivering thermogenesis (Cannon and Nedergaard 2004). If nonshivering thermogenesis is impaired (as in the UCP1-ablated mice (Golozoubova et al. 2006)), the mice will have to utilize other mechanisms to survive longer periods in cold. Although it was speculated that the Cav1-ablated would not survive longer periods in cold, cold-acclimated Cav1-ablated mice were able to survive, and this was accomplished by nonshivering thermogenesis. Similarly to the thyroid-hormone receptor-ablated mice (Golozoubova et al. 2004), Cav1-ablated mice have desensitized adrenergic receptors. However, in both these ablated mice, this could be rescued physiologically, and the mice were able to survive. Thus, Cav1 is not essential for survival in prolonged cold or for nonshivering thermogenesis (paper I).

β3-adrenergic, LPA (lysophosphatidic acid)-, EGF (epidermal growth factor)- and PDGF (platelet-derived growth factor)-receptors have been investigated to different extents with regard to caveolin and caveolae (Pike 2005, Patel et al. 2008a). The receptors have been shown to be colocalized with caveolae (caveolin). However, many of the colocalization studies have not distinguished between caveolae and lipid rafts, and if cofractionation with caveolin was seen, this was regarded as being colocalization with caveolin and caveolae. For the receptor signaling pathways, studies have shown that caveolae/caveolins are important (both stimulatory and inhibitory), but also that caveolae/caveolin do not affect the signaling pathways (see section 5.1,4-6, and below). Whether these differences are due to cell type or methodology issues, the future will hopefully tell us. It should also be stated
that both treatment of cells with methyl-β-cyclodextrin and Cav1 siRNA have been shown to affect Erk1/2 activation without the addition of agonist (Furuchi and Anderson 1998, Engelman et al. 1998, Chen and Resh 2001, Cohen et al. 2003b, Gosens et al. 2006, Liu et al. 2007, Yin et al. 2008, Park and Han. 2009). Thus, both cholesterol and Cav1 can influence Erk1/2 activation.

Both EGF and PDGF can activate Erk1/2 in different cell systems; however, no general pathway has been described (Heldin and Westermark 1999, Corbit et al. 2000, Tsakiridis et al. 2001, Jorissen et al. 2003, Osaki et al. 2004, Hennessy et al. 2005). In brown adipocytes, EGF and PDGF are able to induce Erk1/2 activation that leads to cell proliferation. However, the signaling pathway is different for EGF and PDGF, with the EGF signaling being partially via a Src-pathway, and PDGF signaling via a PI3K/PKC/Src-pathway (paper II).

The involvement of caveolae/lipid rafts in EGF-induced Erk1/2 activation has been investigated with different results. Treatment with cholesterol-disturbing agents (methyl-β-cyclodextrin, nystatin, and filipin) can both increase (Furuchi and Anderson 1998, Pike and Casey 2002), decrease (Kraenenburg et al. 2001, Park and Han 2009) and have no effect (Peiro et al. 2000) on the EGF-induced Erk1/2 activation. Cav1 siRNA reduces EGF-induced Erk1/2 activation in mouse embryonic stem cells (Park and Han 2009), but in hepatic C9 cells, Cav1 siRNA has no effect on EGF-induced Erk1/2 activation. In brown adipocytes isolated from Cav1-ablated mice, Cav1-ablation has no effect on EGF-induced Erk1/2 activation (paper IV). Furthermore, depending on the age of human diploid fibroblasts, different amounts of Cav1 are expressed (increasing with age), and the expression is inhibitory for EGF-induced Erk1/2 activation (Park et al. 2000). Thus, both cholesterol and Cav1 may have an effect on EGF-induced Erk1/2 activation; whether there is an effect or not seems to be cell-type specific and also dependent on the age of the cells.

For PDGF-induced Erk1/2 activation, three studies have investigated the importance of Cav1 for the signaling. In airway smooth muscle cells, Cav1 is required for proper signaling (Gosens et al. 2006), while in rat primary mesangial cells, Cav1 is inhibitory for PDGF-induced Erk1/2 activation. In brown adipocytes isolated from Cav1-ablated mice, Cav1-ablation has no effect on PDGF-induced Erk1/2 activation (paper IV). Thus, in these three cell types, Cav1 influences the PDGF-signaling to Erk1/2 differently.

LPA can activate Erk1/2 in many cell systems, and there is no general pathway. G-proteins, PI3K, PKC, and Src have been implicated in the pathway (Moolenaar. 1999, Anliker and Chun 2004, Shah et al. 2006, Kassel et al. 2008, Jeong et al. 2008) and also transactivation via EGF and PDGF receptor (Daub et al. 1997, Goppelt-Struebe et al. 2000, Rodland et al. 2008). In
primary brown adipocytes, LPA does not induce Erk1/2 activation via EGF or PDGF transactivation. Instead, two non-transactivational pathways exist, one Gi/PKC/Src-pathway and one PI3K-pathway (paper III).

The involvement of caveolae/lipid rafts in LPA-induced Erk1/2 activation has been investigated in two studies. In Cos-7 cells, treatment with methyl-β-cyclodextrin decreases the LPA-induced Erk1/2 activation (Kranenburg et al. 2001). Thus, caveolae/lipid rafts are important for proper signaling. In brown adipocytes isolated from Cav1-ablated mice, however, Cav1-ablation has no effect on the LPA-induced Erk1/2 activation (paper IV). Thus, it is possible that lipid rafts and not caveolae/Cav1 regulate LPA signaling to Erk1/2, although cell type specificity cannot be disregarded.

The involvement of caveolae/lipid rafts in β3-adrenergic receptor (β3-AR) signaling to cAMP has been investigated in three studies. Treatment of brown adipocytes with the cholesterol-disturbing agent filipin reduces β3-AR-induced cAMP levels (Sato et al. 2007). Thus, caveolae/lipid rafts seem to play a role in the signaling. In addition, in brown adipocytes isolated from Cav1-ablated mice, the Cav1-ablation decreased the β3-AR-induced cAMP levels (paper IV). However, in C6 glioma cells, treatment with Cav1 siRNA increased β-AR-induced cAMP levels compared to untreated cells (Allen et al. 2009). Thus, the importance of caveolae/Cav1 (and lipid rafts) is probably cell-type specific.

In conclusion, caveolae and Cav1 can affect the localization of receptors and the receptor signaling. This seem to be cell-type specific. The presence of brown adipose tissue in adult man has been negatively correlated with both obesity and age (van Marken Lichtenbelt et al. 2009, Virtanen et al. 2009, Zingaretti et al. 2009, Saito et al. 2009). By understanding how brown adipocyte proliferation and differentiation are regulated, it could be possible to combat obesity and the metabolic syndrome (see also (Fruhbeck et al. 2009)).
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8. References


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