Thesis for the Master of Science degree in Molecular Biosciences
Main field of study in molecular biology

STAMP2: subcellular localization and effect on synthesis and sulfation of glycans

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Joakim Sorthe

"Focus on the journey, not the destination. Joy is found, not in finishing an activity, but in doing it."

ABSTRACT

Six transmembrane protein of prostate 2 (STAMP2) is a six transmembrane domain protein in the STAMP/STEAP family. It is primarily found in the prostate, visceral adipose tissue, and bone marrow, and is overexpressed in prostate cancer cells. STAMP2 has an oxidoreductase activity and is capable of reducing both iron and copper, and is shown to co-localize with the metal transporters DMT1 and CTR1 in endosomes, where it is thought to reduce iron and copper for translocation across the membrane. STAMP2 is also proposed to be involved in the integration of inflammatory and metabolic responses in mice. A similar role is suggested for STAMP2 in humans, although the exact functions of the protein remain to be determined.

The Saatcioglu group had previously expressed a GFP-tagged STAMP2 variant with significant co-localization with Golgi markers. A pilot study conducted by the Prydz and Saatcioglu groups to monitor Golgi functions had indicated that reduction of STAMP2 expression in LNCaP cells had an effect on the incorporation of sulfate into proteoglycans (PGs). Since copper ions previously have been suggested to play a role in PG metabolism, it was of interest to study this possible link further. With the extensive knowledge of PG synthesis and sulfation in the epithelial cell line MDCK, as well as available methodology for transport and subcellular fractionation studies, it would be of interest to study the impact of STAMP2 in transfected MDCK cells, as well as to further study the link between STAMP2 and synthesis and sulfation of PGs in LNCaP cells.

Three STAMP2 variants fused to GFP were transfected into MDCK II cells. One of these was the original construct from the Saatcioglu group with the GFP at the very N-terminus (N-terminal signal sequence for ER import not required). The two other variants had the GFP moved into the N-terminal region of protein, one of these with a mutated ferric-reductase domain. Confocal microscopy and subcellular fractionation studies indicated a difference in the localization of the three variants. Exposure of the N-terminal cytoplasmic tail of STAMP2 caused localization to the plasma membrane and endosome-like strucutres, while blocking the tail with GFP, resulted in significant localization to the Golgi apparatus. Radioactive labeling of control cells and the transfected cell lines with [^{3}H]-glucosamine and [^{35}S]-sulfate gave a significant decrease in the incorporation of glucosamine and sulfate into GAG chains in the cells where STAMP2 had the GFP domain at the very N-terminus and showed Golgi localization.

The stable knockdown of STAMP1 and STAMP2 in LNCaP cells had no effect on the synthesis and sulfation of glycoproteins.

ABBREVIATIONS

Amp	Ampicillin
AP	Adaptor protein
Api	Apical
AR	Androgen receptor
ATP	Adenosine triphosphate
Baso	Basolateral
BAT	Brown adipose tissue
bp	Base pairs
cABC	Chondroitinase ABC
Cnx	Calnexin
COP	Coat protein complex
CPM	Counts per minute
Crt	Calreticulin
CS	Chondroitin sulfate
Ctrl	Control
Da	Dalton
dH_2O	Destilled water
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
Dmt1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DS	Dermatan sulfate
E. coli	Esherichia coli
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetate
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ERGIC	ER - Golgi intermediate compartment
Et al.	And others (Latin: et alibi)
EXT	Exostosin
FBS	Fetal bovine serum
FRD	Ferric-reductase domain
Fig.	Figure
g	Gram
GAG	Glycosaminoglycan
GalNAc	N-acetyl-galactosamine
GFP	Green fluorescent protein
GH	Growth hormone
Glc	Glucose
GlcA	D-glucuronic acid
GlcNAc	N-acetyl-glucosamine
Gln	L-glutamine
GPI	Glycosylphosphatidylinositol
HA	Hyaluronic acid
HBSS	Hank's balanced salt solution
HS	Heparan sulfate
IdoA	L-iduronic acid
IL-1	Interleukin-1
IP	Immunoprecipitation
Kan	Kanamycin
kDa	Kilo Dalton
kb	Kilo base pairs
KS	Keratan sulfate
LB	Lysogeny broth
LDL	Low-density lipoprotein
M	Molar
mA	Milliampere
Man	Mannose
mCi	Millicurie
MDCK	Madin-Darby canine kidney
MHC	Major histocompatibility complex
ml	Milliliter
mM	Millimolar
N	Nitrogen
nm	Nanometer
O	Oxygen
OD	Optical density
ON	Over night
ORD	Oxidoreductase domain
P/S	Penicillin/streptomycin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGs	Proteoglycans
pmol	Picomole
PNGase F	Peptide: N-Glycosidase F
PNS	Postnuclear supernatant
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid

S2	STAMP2
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
SOB	Super optimal broth
SRP	Signal recognition particle
STAMP	Six transmembrane protein of Prostate
STEAP	Six transmembrane epithelial antigen of the prostate
TAE-buffer	Tris, acetate, EDTA - buffer
TB	Transformation buffer
TBS	Tris buffered saline
Tf	Transferrin
Tfr1	Transferrin receptor 1
TGN	Trans-Golgi network
TIARP	Tumor necrosis factor alpha-induced adipose related protein
TSAP6	Tumor suppressor-activated pathway protein 6
TTBS	Tween tris buffered saline
UDP-xylose	Uridine diphosphate-xylose
UTP	Uridine triphosphate
UV	Ultraviolet
V	Volt
VTC	Vesiculotubular cluster
VTS	Vesiculotubular structures
WAT	White adipose tissue
WB	Western blot
x g	Times gravity
µl	Microliter

1. INTRODUCTION

1.1 Epithelial cells

Our bodies consist of many different types of cells. Epithelial cells are the ones covering the external and internal surfaces of the body, such as the skin, intestine, and kidney tubules. Their structure varies depending on their location and function, but common for them all is that they form a tight cell layer that acts as a selective barrier between the body and the environment, often represented by the lumen of our body cavities. The cells are tightly connected via tight junctions (Gumbiner 1987), a structure that prevents intercellular diffusion of molecules from one side of the cell layer to the other.

The plasma membrane of epithelial cells can be distinguished into three domains. The surface that faces the inner cavities of the body, is called the apical surface. Facing the neighboring epithelial cells is the lateral surface, while the area of the cell facing the body tissues is called the basal surface. Because the basal and lateral surfaces are continuous, these domains are often collectively referred to as the basolateral membrane. The apical and basolateral domains differ in their lipid composition. While the apical domain is enriched in glycosphingolipids, the basolateral domain has a high concentration of phosphatidylcholine. The tight junctions prevent the mixing of lipids and membrane proteins between the two domains, thereby maintaining the difference in composition (Dragsten et al. 1981; van Meer and Simons 1986). Epithelial cells are anchored to the extracellular matrix (ECM), via among others, a class of glycoproteins called integrins (Schoenenberger et al. 1994).

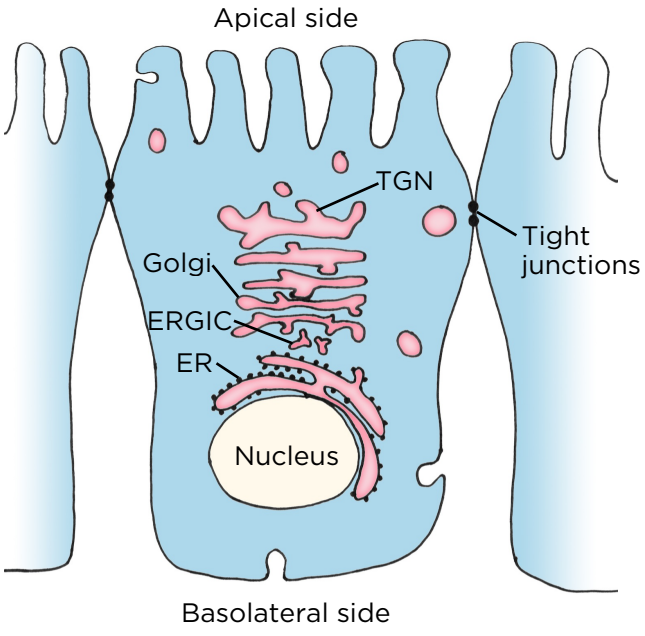

Figure 1.1 - Schematic figure of an epithelial cell
Tight junctions separate the apical domain from the basolateral domain and prevents diffusion of molecules and ions between the two sides of the monolayer. ER = Endoplasmic reticulum, ERGIC = ER Golgi intermediate compartment, TGN = Trans Golgi network

1.1.1 MDCK cells

Madin-Darby canine kidney (MDCK) cells are one of the best characterized and most frequently used cell lines for studying epithelial cells and polarized transport. It was originally isolated from the kidney of an adult female cocker spaniel in 1958. It is a heterogenous cell line, from which different sublines have been derived. There are two different strains of the cell line, MDCK I and MDCK II, with several differences in physiological properties (Barker and Simmons 1981). MDCK II has a lower electrical resistance across the cell layer than MDCK I, indicating a more leaky epithelium, corresponding to proximal tubules. MDCK I resembles more the cells of the collecting duct segments of a kidney (Richardson et al. 1981). The two cell lines also vary in the protein composition of their apical and basolateral cell surface domains. In addition, MDCK I cells have less microvilli apically and a more folded membrane basolaterally than MDCK II cells.

When MDCK cells are grown on permeable filters a polarized monolayer is formed, with morphology and functions similar to those of epithelial cells *in vivo* (Cereijido et al. 1978). These features make MDCK cells a suitable model system for studying polarized transport in epithelia.

1.2 The secretory pathway

Transmembrane proteins of the endomembrane system and the plasma membrane, and also secretory proteins are synthesized by ribosomes on the cytoplasmic side of the endoplasmic reticulum (ER) and translocated into the lumen of the ER. From there the proteins pass several sorting stations on their way to their final destination. It was previously thought that the *trans*-Golgi network (TGN) was the major site for protein sorting, but this view have changed in recent years.

1.2.1 The Endoplasmic Reticulum

Proteins destined for the secretory pathway usually have an N-terminal signal sequence. The signal sequence is recognized by a signal recognition particle (SRP) as the N-terminus of the protein exits the ribosome. The SRP binds tightly to the ribosome and causes pause in the translation of the protein. An SRP receptor in the ER membrane recognizes and binds to the SRP, causing it to be released from the ribosome. The ribosome then binds to other components in the ER membrane to translocate the protein across the membrane as it is synthesized (Walter and Johnson 1994). Inside the ER lumen, the signal sequence is often cleaved off, and translocated proteins may undergo N-linked glycosylation, by the addition of the oligosaccharide $Glc_3Man_9GlcNAc_2$ (described in further detail below) and disulfide bond formation between cysteines, required for proper folding of some proteins. Chaperones enable a more effective folding of proteins, and also contribute to quality control by retaining incorrectly folded proteins within the ER and with time, targeting unfolded proteins for degradation via the ER-associated degradation (ERAD). ER chaperones include members of the Hsp40, Hsp70,

Hsp90, Hsp100, and calnexin/calreticulin (Cnx/Crt) families (Williams 2006). In mammalian cells, Cnx and Crt retain immature glycoproteins and assist their folding. They bind to the terminal glucose of $Glc_1Man_9GlcNAc_2$, after the other two glucose units have been cleaved off. Upon disassociation from the chaperons, the last glucose is removed. If the protein is correctly folded, the oligosaccharide is further trimmed, and the protein is exported to the Golgi apparatus. However, if the protein is incorrectly folded, the glucose is re-added for another round of Cnx/Crt association. Misfolded glycoproteins that remain for prolonged periods in the Cnx/Crt cycle will have a mannose cleaved off to generate $Man_8GlcNAc_2$, which acts as a degradation signal, leading to retrotranslocation and proteasomal degradation in the cytosol (Ellgaard and Helenius 2003; Meusser et al. 2005; Williams 2006). Other proteins are anchored in the membrane by a hydrophobic C-terminal end. This C-terminal end can be cleaved off and replaced by a glycosylphosphatidylinositol (GPI) anchor if the protein contains a GPI signal sequence (Rudd and Dwek 1997).

Exit from the ER is dependent on coat protein complex (COP) II coated vesicles. Soluble cargo proteins can be concentrated in COPII vesicles by binding to COPII components, or to sorting receptors for transport to the Golgi apparatus (Kuehn et al. 1998; Belden and Barlowe 2001).

1.2.2 The ERGIC

The ER - Golgi intermediate compartment (ERGIC, also known as intermediate compartment (IC), or vesiculotubular cluster (VTC)), is a membrane cluster identified by the lectin ERGIC-53, located between the ER and Golgi apparatus. ERGIC exhibits different properties from both ER and cis-Golgi (Schweizer et al. 1991). The dynamic nature and function of ERGIC have been debated, although the most popular current view is that it is a stationary sorting compartment for both anterograde transport to the Golgi apparatus in COPII coated vesicles, and retrograde transport back to the ER in COPI coated vesicles (Appenzeller-Herzog and Hauri 2006).

The specific composition of proteins in different compartments is maintained by active transport. An example is the KDEL sequence found in the C-terminal end of many soluble ER resident proteins and some membrane proteins. ER proteins with the KDEL sequence associate with KDEL receptors in the Golgi apparatus, before transport back to the ER in COPI coated vesicles (Pelham 1996). The binding of the KDEL sequence to the KDEL receptor is pH dependent, allowing the receptor to bind to proteins with this sequence in the slightly acidic Golgi (pH 6.2) and release them in the neutral ER (pH 7.4) (Wilson et al. 1993; Wu et al. 2001; Appenzeller-Herzog et al. 2004). The KDEL receptors are recycled back to the Golgi by COPII coated vesicles. Several variants of the KDEL sequence have been shown to retrieve ER resident proteins (Raykhel et al. 2007).

1.2.3 The Golgi apparatus

The Golgi apparatus is an organelle in eukaryotic cells first described by Camillo Golgi. The Golgi is composed of flat, membrane enclosed *cisternae*, forming stacks. Although the organization of Golgi stacks differs between cell types, it is generally located in the perinuclear area in mammalian cells (Farquhar and Palade 1981). The Golgi apparatus has two main roles. One is modification of newly synthesized proteins and lipids by addition of carbohydrate groups, phosphates, sulfates or other alteration, as they pass through the organelle. Proteins may also be proteolytically cleaved. The Golgi apparatus also serves as a major sorting site in the secretory pathway, selectively targeting proteins and lipids to several different membrane systems (Munro 1998).

Each Golgi stack can be divided into three, the *cis*-, *medial*- and *trans*-Golgi compartments, with different enzyme and membrane compositions (Munro 1998). There were two proposed models for anterograde transport through the Golgi apparatus: The vesicle transport model, where cargo proteins are transported from one Golgi compartment to the next by COPI vesicles and/or VTC-like structures, and the cisternal maturation model, where entire *cisternae* carry the secretory cargo forward, and retrograde COPI vesicles recycle resident Golgi proteins to "younger" *cisternae*. It has also been suggested that the two different principles could work in combination (Glick 2000; Pelham and Rothman 2000). More recent studies include observations of cisternal connections (Pelham 2006) and the Lippincott-Schwartz model, where the Golgi acts as a transport apparatus, involving membrane partitioning rather than a progressing set of *cisternae* (Patterson et al. 2008).

1.3 Polarized sorting

The apical and basolateral domains of an epithelial cell have different lipid and protein composition. The differences are maintained by polarized transport of lipids and proteins.

1.3.1 Basolateral sorting signals

Basolateral sorting signals are usually located to the cytoplasmic domain of basolateral proteins, and do often consist of tyrosine or dileucine motifs, often with nearby acidic amino acids (Rodriguez-Boulan et al. 2005). Tyrosine-based basolateral sorting signals contain a tyrosine in a NPXY or YXXφ motif (where φ is a hydrophobic amino acid) and are often overlapping with signals for localization to coated pits in clathrin-mediated endocytosis (Muth and Caplan 2003). Endocytic, recycling, and basolateral sorting signals all interact with a family of adaptor proteins (AP). AP1, AP3, and AP4 mediate sorting in the TGN and endosomes, while AP2 functions in sorting into endocytic clathrin coated vesicles at the plasma membrane. AP1, AP3 and AP4 are all involved in sorting of basolateral membrane proteins (Simmen et al. 2002). AP1B has been shown to promote the basolateral sorting of the low-density lipoprotein (LDL) and transferrin receptors in recycling endosomes (Gan et al. 2002). AP1A localizes to the TGN, and is involved

in the sorting of mannose-6-phosphate receptors from the TGN to endosomes (Rodriguez-Boulan et al. 2005).

1.3.2 Apical sorting signals

The first apical sorting signal was the GPI anchor. Addition of a GPI-anchor to proteins not previously sorted to the apical membrane, resulted in apical localization of these proteins (Lisanti et al. 1989; Gut et al. 1998; Rodriguez-Boulan et al. 2005; Paladino et al. 2006). The transport of GPI-anchored proteins to the apical surface seemed to be dependent on lipid rafts (Simons and Ikonen 1997; Muth and Caplan 2003). Subsequent analysis has shown that not all proteins are sorted apically upon addition of a GPI-anchor (Muth and Caplan 2003). Another signal shown to mediate apical sorting of some proteins, is N-glycans (Martínez-Maza et al. 2001), although studies have shown that for some apical proteins, N-glycosylation does not contribute to the apical sorting (Marzolo et al. 1997). In addition, both O-glycans and proteinaceous motifs in the cytoplasmic or transmembrane region of proteins have been shown to be able to mediate apical sorting of proteins(Marzolo et al. 2003; Takeda et al. 2003; Rodriguez-Boulan et al. 2005).

Both apical and basolateral trafficking signals promote the sorting of cargo proteins into different vesicles in the TGN, at the plasma membrane or in recycling endosomes. Membrane proteins lacking a signal for forward transport, accumulate in the Golgi apparatus and are delivered quite inefficiently to the plasma membrane, indicating that efficient exit from the Golgi complex requires a sorting signal (Gut et al. 1998).

The delivery of vesicles to the correct target membranes is regulated by SNAREs. The cargo vesicles contain v-SNAREs that can only interact with t-SNAREs at the proper target membrane. In addition to the SNAREs, Rabs are also important regulators of vesicle traffic and docking (Novick and Zerial 1997; Muth and Caplan 2003).

1.4 Proteoglycans and Glycoproteins

1.4.1 Proteoglycans

Proteoglycans (PGs) belong to a diverse family of macromolecules, composed of one or several glycosaminoglycan (GAG) chains covalently attached to a protein core. The protein core can have more than hundred GAG chains attached. Newly synthesized PGs are transported to the plasma membrane, where they may be secreted, attach cells to matrix components via their GAG chains and transmembrane domains, or become endocytosed. Their functions range from mechanical support in the ECM to effects in different cellular processes such as cell adhesion, motility, and proliferation (Kjellén and Lindahl 1991; Kolset et al. 2004). The large extracellular PG versican, for example, has been shown to play a role in cell adhesion, cell proliferation, cell migration and ECM assembly (Zimmermann and Ruoslahti 1989; Wight 2002).

GAG chains are normally composed of unbranched, sulfated, repeating disaccharide units of a hexosamine (either N-acetyl-Glucosamine (GlcNAc) or N-acetyl-Galactosamine (GalNAc)) and hexuronic acid (either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)). On the basis of their sugar composition, there are four main types of GAG chains: Chondroitin sulfate (CS), dermatan sulfate (DS), heparan sulfate (HS) and heparin. In addition, there is keratan sulfate (KS), which has a galactose instead of a hexuronic acid, and hyaluronic acid (HA), which is not attached to a protein core, but exists in an unsulfated, free form in the ECM. Some PGs can carry different types of GAG chains, and are then called hybrids (Rapraeger et al. 1985). Others are called part-time PGs, since they can be synthesized both with and without GAG chains. Examples are MHC class II invariant chain and thrombomodulin (Fransson 1987).

1.4.2 Synthesis of the linker region

The synthesis of a GAG chain (with the exception of KS and HA) begins with four sugars attaching sequentially to a serine, in a serine-glycine motif in a protein core. The tetrasaccharide is composed of a xylose, followed by two galactoses and a glucoronic acid (Figure 1.2). The process is catalyzed by several enzymes. Xylosyltransferase I or II initiates the synthesis by transferring xylose from uridine diphosphate-xylose (UDP-xylose) to the serine residue (Götting et al. 2000). Then galactosyltransferases I and II are responsible for attachment of the first and second galactose units, respectively (Almeida et al. 1999; Bai et al. 2001), before the glucuronic acid is attached by a glucuronyltransferase (Kitagawa et al. 1998). The linker region of both CS and HS GAG chains are synthesized by the same set of enzymes (Bai et al. 1999). The addition of xylose to a serine occurs in a pre-Golgi compartment (Kearns et al. 1993), while the growth of the linker region occurs in the *cis* or *medial* Golgi (Silbert and Sugumaran 1995). The linker region can be modified by both phosphorylation and sulfation. The C-2 of the xylose is a major phosphorylation site on both CS chains (Oegema et al. 1984) and HS chains (Fransson et al. 1985). Sulfation on the other hand, has only been observed on CS/DS chains, on the second galactose (Sugahara et al. 1988).

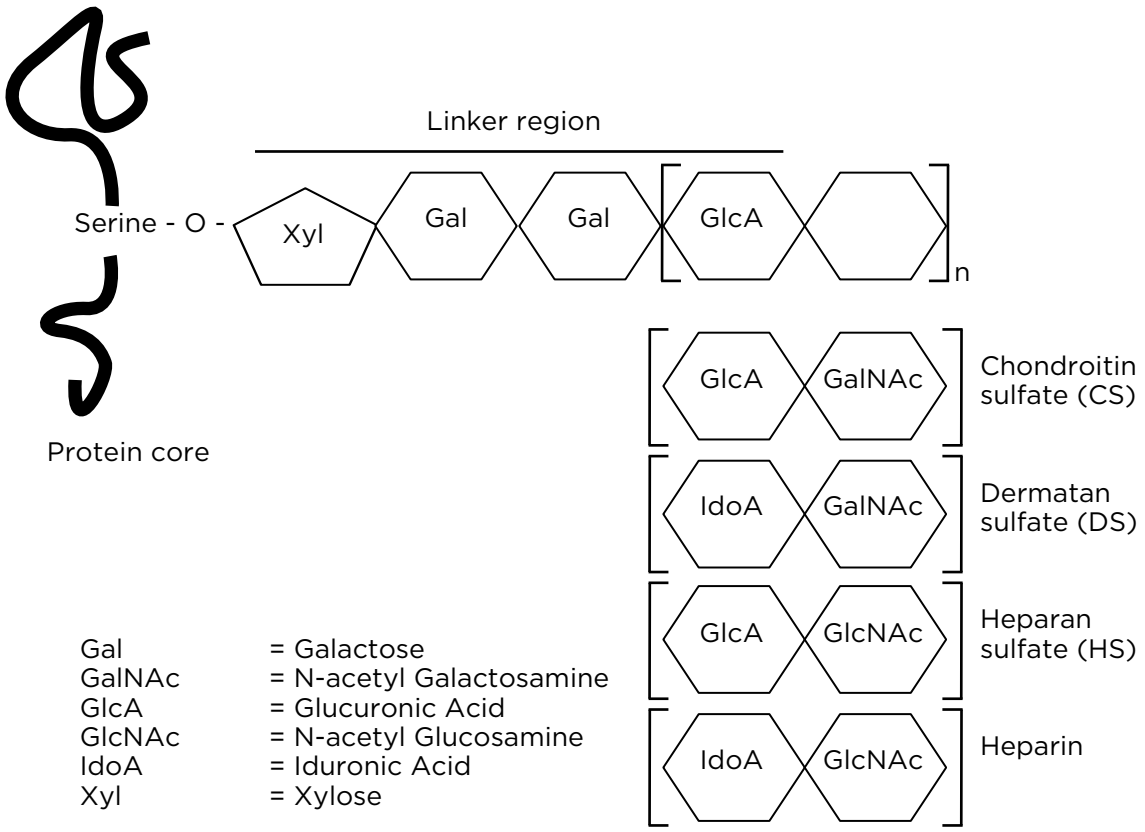

Figure 1.2 - Composition of linker region

1.4.3 Synthesis of GAG chains

After completion of the linker tetrasaccharide, the addition of the fifth sugar determines whether the GAG chain becomes CS/DS or HS/heparin (Prydz and Dalen 2000). The polymerization of HS and CS are catalyzed by different enzymes located in different parts of the Golgi apparatus. In CS/DS biosynthesis, the fifth sugar is GalNAc, and CS synthases will subsequently add GlcA and GalNAc in an alternating sequence (Prydz and Dalen 2000; Kitagawa et al. 2001). In HS biosynthesis, the fifth sugar is GlcNAc, which is followed by the addition of GlcA and GlcNAc by the glycosyltransferases EXT1 and EXT2 (Lind et al. 1998). The enzymes required for the biosynthesis of HS chains are localized in the proximal part of the Golgi complex, whereas the enzymes involved in the elongation and sulfation of CS chains are located in the trans-Golgi network (TGN) (Spiro et al. 1991; Uhlin-Hansen and Yanagishita 1993; Calabro and Hascall 1994). The growing GAG chains may undergo modification at several different positions: The GlcNAc units in HS and heparin can be deacetylated and N-sulfated, GlcA can be epimerised to IdoA in HS, heparin and DS, and the disaccharides of HS, heparin, CS, and DS can be O-sulfated in various positions (Prydz and Dalen 2000). The sulfation is catalyzed by sulfotransferases that use 3'-Phosphoadenosine 5'-Phosphosulfate (PAPS) as donor (Kusche-Gullberg and Kjellén 2003). The degree of epimerisation of GlcA to IdoA, and the sulfation pattern of the disaccharides distinguish heparin from HS, and DS from CS (Prydz and Dalen 2000).

At the cell surface, PGs are involved in recruitment of several classes of signaling molecules, like growth factors and cytokines, that bind to the negatively charged GAG chains. Thus the sulfation pattern of GAG chains and their abundance at the cell surface is important for the tuning of signaling processes. PGs are often actively endocytosed after reaching the cell membrane (Yanagishita and Hascall 1992). One example is the GPI-anchored HS containing PG glypican-1. Glypican-1 is recycled via endosomes, where he HS chains are degraded in a copper ion dependent process, and further to the Golgi apparatus where GAG chains are resynthesized (Mani et al. 2000; Ding et al. 2002). All the intracellular compartments in this recycling route have not been identified, but the resynthesis of HS chains implies recycling to an early Golgi compartment. The endocytosis of PGs may be important for many aspects of cellular signaling processes. PG-mediated internalization of for instance fibroblast growth factors and polyamines is important for proliferation and growth control (Kolset et al. 2004). Possibly both for appropriate down-regulation of the signal, but GAG chains have also been observed in the nucleus and PGs might be involved in nuclear import of regulatory proteins that entered the cell via endocytosis.

1.4.4 Glycoproteins

There are two main types of glycans attached to glycoproteins, N-linked and O-linked glycans. In N-linked glycosylation oligosaccharide chains are attached to aspargines in N-X-S/T motifs, where X may be any amino acid, except proline. N-glycosylation is a co-translational modification that starts in the endoplasmic reticulum (ER), where the N-glycans are synthesized on a dolichol diphosphate in the cytoplasmic leaflet of the ER membrane and flipped at an intermediate stage. A core structure composed of $Glc_3Man_9GlcNAc_2$, is built sequentially by glycosyltransferases. The transfer of the precursor to the Asn side chain takes place during translocation of the polypeptide into the ER, before protein folding is complete. When the protein is fully folded, the three terminal glucose residues are removed. The glycoprotein is then transported to the Golgi apparatus, where removal of mannose residues may take place, before addition of different monosaccharides, including GlcNAc, GalNAc, galactose, fucose, and sialic acid. As seen in figure 1.3 there are three main classes of N-glycans, depending on the composition of sugars: High mannose, complex, and hybrid (Rudd and Dwek 1997; Varki et al. 1999).

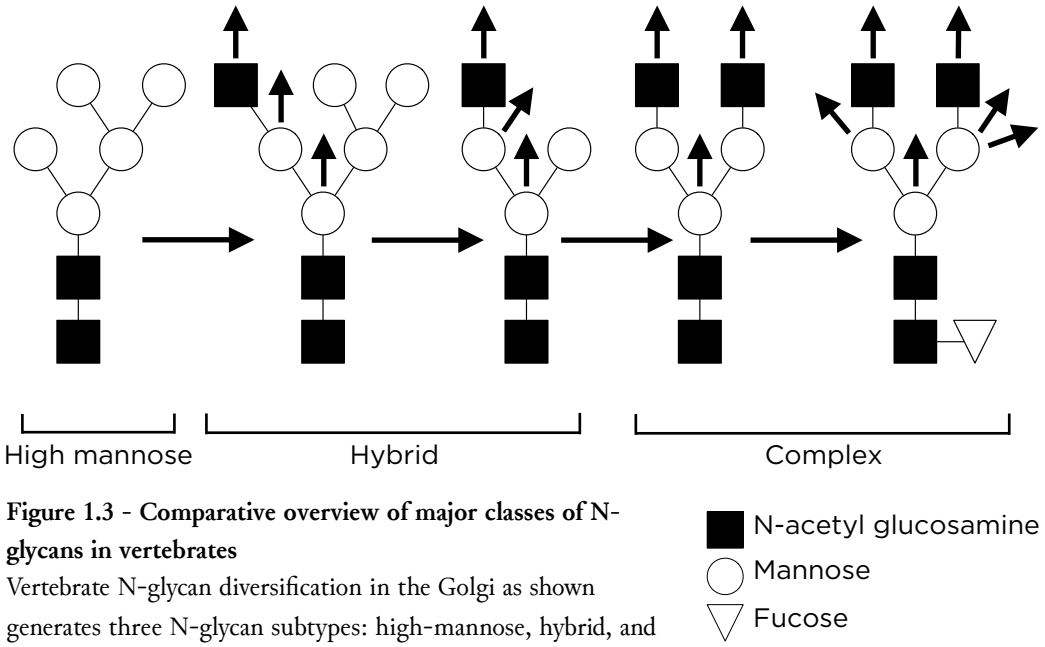

Figure 1.3 - Comparative overview of major classes of N-glycans in vertebrates
Vertebrate N-glycan diversification in the Golgi as shown generates three N-glycan subtypes: high-mannose, hybrid, and complex. Most secreted and cell surface N-glycans are of the complex type. Arrows indicate locations of branch formation in diversification, not all of which occur on a single N-glycan. Figure adapted from Varki et al. (1999).

O-linked glycosylation is normally initiated in the Golgi apparatus, usually by an N-acetyl galactosaminyltransferase that transfers a GalNAc to the oxygen of a serine or threonine side chain. Additional monosaccharides are subsequently added by various transferases (Steen et al. 1998).

1.5 The STAMP/STEAP proteins

The six transmembrane protein of prostate (STAMP) family is a protein family in vertebrates that consist of four members: Six transmembrane epithelial antigen of the prostate 1 (STEAP1), STAMP1 (also known as STEAP2), STAMP2 (also known as STEAP4 and tumor necrosis factor alpha-induced adipose related protein (TIARP) (mouse homologue)) and STAMP3 (also known as STEAP3, tumor suppressor-activated pathway protein 6 (TSAP6) or pHyde (rat homologue)). All members of the family have the six transmembrane domains, a short cytoplasmic C-terminal domain, and, except for STAMP1, they all have a long cytoplasmic N-terminal region containing a domain predicted to have NADP+/NADPH oxidoreductase activity (figure 1.4) (Korkmaz et al. 2005; Ohgami et al. 2005). STAMP1, STAMP2, and STAMP3 are all metalloreductases capable of reducing both iron and copper, by a ferric-reductase domain in the C-terminal region. In addition, these proteins also stimulate the uptake of iron and copper into cells (Ohgami et al. 2006). STEAP1, STAMP1, STAMP2, and STAMP3 have all been shown to co-localize with transferrin (Tf), transferrin receptor (Tfr1), and divalent metal transporter 1 (DMT1)(Ohgami et al. 2005). STAMP2 and STAMP3 are

postulated to reduce iron dissociated from Tfr1 for translocation into the cytosol by DMT1 (Knutson 2007).

STEAP1, STAMP1, and STAMP2 are all overexpressed in prostate cancer cells (Hubert et al. 1999; Korkmaz et al. 2002; Korkmaz et al. 2005). In addition, STEAP1 is also up-regulated in several other cancer cell lines (Hubert et al. 1999). STAMP3 has been shown to form homodimers via a dimerization domain in the N-terminal oxidoreductase domain. The conservation of the dimerization domain within the STAMP family, with the exception of STEAP1, suggests that other members of the STAMP family can form dimers (Sendamarai et al. 2008). STEAP1, STAMP1, and STAMP2 have all been implicated as positive regulators of proliferation and survival of cancer cells (Porkka et al. 2002; Korkmaz et al. 2005; Challita-Eid et al. 2007), while STAMP3 has been shown to inhibit growth of cancer cells, in part through the induction of apoptosis (Steiner et al. 2000; Zhang et al. 2001).

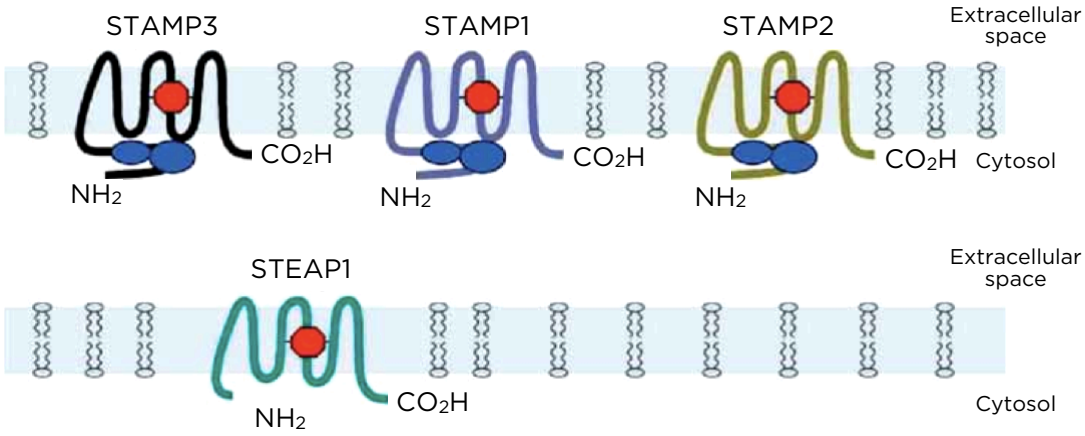

Figure 1.4 - Transmembrane structure of the STAMP/STEAP family
Schematic diagram of transmembrane structure of Steap1–Steap4. Blue ovals in tandem represent the flavin-NAD(P)H binding oxidoreductase domain. Heme groups are indicated in red. Adapted from Ohgami et al. (2005).

1.5.1 STAMP1

STAMP1 (STEAP2) was identified while searching for genes that are differentially expressed during early stages of prostate cancer (Korkmaz et al. 2002). The expression has been shown to be highly prostate specific, with an expression level ten times higher in normal prostate tissue, compared to other tissues studied, and more than two times higher in cancerous prostate compared to normal glands (Korkmaz et al. 2002; Porkka et al. 2002). STAMP1 has been shown to localize to the Golgi (primarily the TGN), plasma membrane, and vesiculotubular structures (VTS), suggesting a possible role in secretory and endocytic pathways (Korkmaz et al. 2002). STAMP1 is highly expressed in the androgen responsive prostate cancer cell line LNCaP, but not in the androgen receptor (AR) negative prostate cancer cell line. Because the expression

of STAMP1 is not regulated by androgen, it would suggest that STAMP1 is dependent on AR and not androgen (Korkmaz et al. 2002).

1.5.2 STAMP2

Unlike STAMP1, STAMP2 has a more general tissue distribution, with high levels in white and brown adipose tissue (WAT and BAT) bone marrow, placenta, heart, lung, liver, and prostate, with a substantially lower expression also seen in liver, skeletal muscle, pancreas, testis, and small intestine. The subcellular localization of STAMP2 is similar to STAMP1, with a primary localization in the TGN, plasma membrane, and VTS (Korkmaz et al. 2005; Ohgami et al. 2006; Wellen et al. 2007). STAMP2 is overexpressed in prostate cancer and regulated by androgen in the AR positive cell line LNCaP. STAMP2 expression could not be detected in AR-negative prostate cancer cell lines, DU145 and PC3. STAMP2 transfected DU145 and PC3 cell lines showed an increase in proliferation and colony formation, indicating a possible role in cell proliferation for STAMP2 (Korkmaz et al. 2005).

TIARP (a mouse homologue of STAMP2) is induced upon adipocyte differentiation and in response to growth hormone (GH), and the inflammatory cytokines: tumor necrosis factor alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6)(Moldes et al. 2001; Fasshauer et al. 2003; Fasshauer et al. 2004; Kralisch et al. 2009). TIARP has been shown to localize at the plasma membrane, co-localizing with caveolin-1. In addition, it contains two possible caveolin-1 binding motifs, indicating a possible interaction with caveolar structures that play a crucial role in insulin signaling, diabetes, glucose and lipid homoeostasis (Moldes et al. 2001; Chambaut-Guérin and Pairault 2005). The role of STAMP2 has been speculated to be involvement in modulation of inflammatory responses and protection of metabolic functions in adipocytes. Treatment of adipocytes with high serum concentrations and fatty acids markedly induced STAMP2 expression to a level comparable to that observed upon TNF-α treatment. STAMP2 was also induced in the visceral white adipose tissue in lean mice during feeding, an effect not seen in genetically obese, leptin-deficient mice, and in mice with a high-fat diet-induced obesity. Knockdown of STAMP2 in adipocytes impairs insulin action and disrupts glucose transport, while STAMP2$^{-/-}$ mice have significantly elevated levels of inflammatory factors in the visceral WAT and develops metabolic diseases on a regular diet (Wellen et al. 2007). The role of STAMP2 in obesity and metabolic disease in humans is a more complicated issue. Two separate papers with contradicting conclusions were published in 2008. One study found that STAMP2 was significantly downregulated in the adipose tissue of obese patients (Zhang et al. 2008), while the other found that the STAMP2 levels were increased in the adipose tissue of obese patients (Arner et al. 2008). Both studies implicated a role for STAMP2 in obesity and metabolic diseases, but further studies are required to clarify the role of STAMP2.

Aims of the study

STAMP2 is a transmembrane protein with a reported iron and copper reductase activity, that is overexpressed in prostate cancer cells and visceral adipose tissue. A STAMP2 fusion protein with GFP N-terminally has been shown to localize to the Golgi apparatus, at the plasma membrane and in VTS in COS-1 cells. The subcellular localization had previously triggered a pilot study in LNCaP cells which indicated that STAMP2 expression could have an impact on post-translational modifications taking place in the Golgi apparatus, notably the incorporation of sulfate into proteoglycans. These preliminary study triggered the interest in expressing variants of STAMP2 fused to green fluorescent protein (GFP) in MDCK cells, where the knowledge of proteoglycan polymerization and sulfation is more extensive, and the methodology for isolation of Golgi fractions has been well established. Sorting signals have previously been found in the cytoplasmic tail of transmembrane proteins. With GFP fused to the N-terminal end of STAMP2, a sorting signal might be blocked, preventing correct localization of the protein. Therefore, the first goal of this study was to investigate the subcellular localization of three STAMP2 variants in transfected MDCK II cells: GFP-S2 (STAMP2 with GFP N-terminally), S2-GFP (STAMP2 with GFP 20 amino acids downstream from the N-terminal end), and S2'-GFP (STAMP2 with GFP 20 amino acids downstream from the N-terminal end and a three amino acid deletion in the ferric-reductase domain in the C-terminal region of STAMP2). Unpublished experiments have (as mentioned above) demonstrated that reduction of STAMP2 expression by siRNA in LNCaP, caused a reduction in the amount of incorporated sulfate in the cells. Our group has established several useful methods for studies of synthesis and sulfation of glycoproteins and proteoglycans in epithelial MDCK cells. Thus, it was a major goal to study the effect of STAMP2 variants on the synthesis and sulfation of glycoproteins and proteoglycans in transfected MDCK cells, while a related goal was to further study the effect of knocking down STAMP2, and also STAMP1, on the synthesis and sulfation of glycans in LNCaP.

2. METHODS

The method section covers all techniques used during the experimental work. Information on instruments, buffers, solutions, reagents, and other materials used can be found in the appendices.

2.1 Working with microorganisms

When working with microorganisms it is important to use sterile solutions, equipment and proper sterile technique to avoid contamination. All solutions and equipment used were either purchased sterile, or subjected to sterile filtration or autoclaved for 20 minutes at 121 °C before use.

The bacterial strain *E. coli* DH5α was used throughout the bacterial work. *E. coli* is one of the best studied prokaryotic model organisms and a widely used bacteria in biotechnology and microbiology.

2.1.1 LB agar plates

LB agar plates are used for selective growth of bacteria. The LB medium contains all the nutrients bacteria needs to grow and amplify. By adding antibiotics to the LB medium it is possible to select for bacteria with inserted foreign DNA. The inserted DNA plasmid contains a gene encoding resistance for a specific antibiotic, and bacteria without the plasmid will not be able to grow in the presence of antibiotic.

Procedure:

- Make LB medium with 1.5 % bactoagar and autoclave it.
- Cool the solution to approximately 50 °C before adding antibiotics.
- Pour the warm medium into plastic dishes. Fill them halfway up and try to avoid formation of air bubbles.
- Let the agar set.
- Store the dishes inverted in sealed bags at 4 °C.

2.1.2 Making *E. coli* freeze culture

For long-term storage of bacterial cultures, the bacteria are frozen at -80 °C in the presence of 15% glycerol to prevent formation of ice crystals.

Procedure:

- Mix the following in a cryovial:
  - 850 µl bacterial culture
  - 150 µl glycerol
- Store at -80 °C.

2.1.3 Growing *E. coli* from freeze culture

Remove a cryovial with freeze culture and place on ice. Use a sterile pipette tip to scrape a small amount of the freeze culture and transfer it to a 50 ml centrifuge tube containing 5 ml LB-medium. The bacteria are cultured at 37 °C for 16 hours with vigorous shaking.

2.1.4 Transformation of *E. coli* cells

In order for bacteria to take up extracellular DNA from the environment, it has to be competent. Bacteria that are not naturally competent, can have competence induced by treatment with $CaCl_2$ and DMSO. For transformation, cells and DNA are mixed and incubated on ice. A short heat shock will make the plasma membrane permeable to the DNA. To identify positive transfectants, the bacteria are plated on LB plates with a selection agent like ampicillin.

2.1.4.1 Induction of competence in *E. coli* cells

This method produces competent *E. coli* cells with high transformation potential.

Procedure:

- Make a preculture by transferring 10-12 fresh bacterial colonies to 100 ml SOB medium in a 500 ml erlenmeyer flask. Incubate at 37 °C for 3-4 hours.
- Measure OD at 600 nm and calculate the amount of preculture needed for an OD of 0.05 in 250 ml of SOB medium.
- Incubate for 16-18 hours at 18 °C (OD_{600} should be about 0.3 - 0.6)
- Incubate the bacterial culture on ice for 10 minutes, before transferring to sterile 50 ml tubes.
- Centrifuge at 2500 x g for 10 minutes at 4 °C.
- Remove supernatants and resuspend bacterial pellets in 80 ml cold transformation buffer (TB).
- Incubate on ice for 10 minutes before centrifugation at 2500 x g for 10 minutes at 4 °C.
- Remove supernatants and resuspend bacterial pellets in 20 ml cold TB.
- Add 700 µl DMSO to each pellet, mix carefully and leave on ice for 5 minutes.
- Add another 700 µl DMSO, mix carefully and leave on ice for 10 minutes.
- Aliquot 200 µl of the resuspended bacterial culture into microfuge tubes and flash freeze in liquid nitrogen. Store at -80 °C.

2.1.4.2 Transformation of competent *E. coli* cells

Procedure:

- Competent cells are thawed on ice.

- Add the plasmid solution to the cells and mix carefully. Leave on ice for 30 minutes.
- Heat shock bacteria at 42 °C for 45 seconds.
- Return to ice for 2-3 minutes.
- The cells are transferred to LB agar plates, with the antibiotic corresponding to the resistance gene of the plasmid, and spread using a sterile glass rod.
- Incubate at 37 °C over night (ON).

2.1.5 Growing bacterial cultures from LB agar plates

Use a sterile pipette tip to pick a colony from the LB agar plate and drop the tip into a 50 ml centrifuge tube containing LB medium with antibiotic. Incubate with vigorous shaking for 16 hours at 37 °C.

2.2 Working with DNA

2.2.1 Quick Check

Quick Check is a method for quickly determining whether plasmid uptake has occurred. The cells are first ruptured by phenol/chloroform. Proteins and chromosomal DNA are denatured by phenol and precipitated in the organic phase, while plasmids remain in the aqueous phase. Chloroform is added to the phenol to more easily to distinguish the phases.

Procedure:

- Mix the following in a microfuge tube:
  - 100 µl of bacteria.
  - 50 µl of phenol/chloroform (1:1).
  - 10 µl of 6X loading dye.
- Vortex mixture for 10 seconds.
- Centrifuge at 10.000 x g for 3 minutes at room temperature.
- Apply 30 µl of the aqueous phase onto an agarose gel (Section 2.2.3)

2.2.2 Isolation of plasmids by Miniprep

Miniprep is based on the alkaline lysis method invented by Birnboim and Doly (1979). Using a NaOH/SDS buffer the bacteria are lysed in alkaline conditions. The membrane dissolves and proteins and chromosomal DNA are denatured. Addition of a neutralization buffer with high salt concentration causes the proteins and chromosomal DNA to precipitate. The supernatant is transferred to a column with a silica membrane, which binds the plasmid DNA. Wash steps will remove salts and remaining cellular components, before elution of the plasmid with a Tris buffer.

Plasmid isolation was carried out using miniprep kits from both QIAgen and Machery Nagel according to protocol, except during elution, where the elution volume was reduced to obtain a higher plasmid concentration.

2.2.3 Gel electrophoresis

Agarose is a linear polysaccaride derived from seaweed. Agarose gels can be used to separate DNA fragments according to size and shape. When an electric current is applied across an agarose gel, DNA fragments will travel towards the positive electrode due to their net negative charge. Shorter fragments will travel faster than longer ones, and circular fragments will travel faster than linear ones.

Procedure:

- Make a 0.8% agarose gel:
  - Add 0.6 g agarose in 75 ml 1X TAE buffer.
  - Heat solution (>50 °C) until agarose is dissolved.
  - Add 3 µl ethidium bromide when the solution has cooled down to about 50 °C.
  - Pour into gel cast and let it set for 30 minutes.
- Add 6X loading dye to samples.
- Run gel at 100 V for 30-45 minutes.
- DNA bands can be visualized using UV light.

2.2.4 Purification of DNA fragments from agarose gel

When purifying DNA fragments from an agarose gel, the piece of agarose containing the DNA fragment is first dissolved in a buffer by heating. The solution is then transferred to the membrane column, where DNA will bind to the silica membrane. Impurities such as agarose, salts, ethidium bromide and others are washed away, before eluting the DNA with a Tris buffer or water.

Purification of DNA fragments from agarose gel was carried out using both QIAquick® Gel Extraction Kit (QIAGEN) and NucleoSpin® Extract II (Machery Nagel) according to the manufacturer's protocol.

2.2.5 Quantification of DNA

DNA was quantified using a NanoDrop™ ND-1000 spectrophotometer according to the manufacturer's protocol. DNA and RNA absorb ultraviolet light, with a peak at 260 nm. The equipment can also determine the purity of the sample by measuring the ratio between the absorption at 260 nm and 280 nm. Proteins, especially the aromatic amino acids, absorb ultraviolet light with a peak at 280 nm.

2.3 Molecular subcloning

2.3.1 Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a method for amplification of specific DNA sequences *in vitro*, by using primers that are complementary to the flanking segments of the target sequence. Through multiple repetitions of the PCR cycle, the DNA fragment will be amplified exponentially.

The PCR consists of three steps, usually repeated 20-40 times:

1. Denaturation at 94 °C, where the hydrogen bonds between the two DNA strands are broken and separated.
2. Annealing of the primers to their complementary DNA strand. The temperature of this step depends on the primer.
3. Elongation at 68 or 72 °C, depending on the DNA polymerase. A DNA-polymerase elongates primers in the 5' - 3' direction, making a complementary DNA sequence.

Because the DNA polymerase is heat-stable it is not necessary to add new enzyme after each cycle. The Advantage® 2 Polymerase Mix (Clontech) used, consists of a mixture of TITANIUM™ Taq DNA polymerase and a proofreading polymerase with 3'-5' exonuclease activity.

Procedure:

- Add the following to a 0.2 ml PCR tube:
  - 2.0 µl DNA-template.
  - 1.0 µl Fwd. primer (20 pmol/µl).
  - 1.0 µl Rev. primer (20 pmol/µl).
  - 1.0 µl of dNTP mix (10 mM).
  - 40.0 µl dH_2O.
- When you are ready to start the reaction, add the following:
  - 5.0 µl PCR buffer.
  - 1.0 µl Advantage 2 Polymerase Mix.

dNTP mix: 10 µl aliquots of each nucleotide (100 mM) are mixed together with 60 µl of dH_2O resulting in 100 µl of mixture with a final concentration of 10 mM for each nucleotide.

PCR program:

1. 94 °C for 2 minutes
2. 94 °C for 30 seconds

3. 56 - 65 °C gradient for 30 seconds
4. 68 °C for 2 minutes
5. Goto 2 for 29 cycles
6. 68 °C for 10 minutes
7. 4 °C forever

The PCR products are applied onto a 0.8 % agarose gel and then cut out and isolated using a gel extraction kit.

2.3.2 Restriction analysis of DNA

Restriction endonucleases are enzymes capable of cutting double or single stranded DNA at specific DNA sequences called restriction sites. Restriction sites are usually short (4-8 bp) and often palindromic.

Procedure:

- Mix the following in a microfuge tube:
  - 5-10 µl plasmid DNA.
  - 0-5 µl MilliQ water.
  - 1.2 µl reaction buffer (10X).
  - 0.5-1 µl restriction enzyme.
- Incubate for 1 hour at 37 °C.

When doing a double digest, make sure to check the manufacturer's website for the optimal buffer.

2.3.3 Ligation

DNA fragments can be joined by DNA ligases that create phosphodiester bonds between the 3' hydroxyl end of one nucleotide and the 5' phosphate end of another nucleotide. When inserting a PCR fragment into a vector, the ratio between vector and fragment can determine the success rate and may require optimization for achieving the best possible result.

Procedure for ligation using an pEGM®-T Easy (Promega) vector:

- Mix the following in a microfuge tube:
  - 10 µl of purified PCR product (Volume depends on size of fragment and concentration).
  - 10 µl of 2X ligation buffer.
  - 1 µl of pEGM-T Easy vector.
  - 1 µl of T4 DNA ligase.

- Incubate for 1 hour at room temperature or ON at 4 °C. Then transform the ligation mixture into *E. coli* cells and plate on agar plates with ampicillin and incubate at 37 °C for 16 hours.

2.4 Sequencing

All the DNA sequencing were performed by the ABI-lab at the University of Oslo. Sample preparation for sequencing of plasmids:

- Mix 8 µl of template (20-100 ng/µl), with 2 µl of a 5 µM primer.

2.5 Mammalian cell work

MDCK II cells are polarized epithelial cells that form a single cell layer with tight junctions. MDCK II cells were grown in 75 cm^2 cell culture flasks with 20 ml Dulbecco's modified Eagle's medium (DMEM) containing 5 % FBS, 1 % L-glutamine and 1 % penicillin and streptomycin (P/S) (Referred to as growth medium from now on, any variations will be noted).

LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells.

All cell work was performed in a sterile cell culture hood using sterile solutions preheated to 37 °C.

2.5.1 Thawing cells

Remove a cryo vial with cells from liquid nitrogen or -80 °C freezer and thaw in a water bath at 37 °C, before transferring cells to cell flasks with 20 ml growth medium.

2.5.2 Trypsination of confluent cells

Tight junctions are protein complexes that seal together epithelial cells in a mono layer and are dependent on Ca^{2+} ions. Addition of EDTA, which binds Ca^{2+}, induces opening of the tight junctions. This allows trypsin to reach the basolateral side of confluent layers of epithelial cells and break the bonds to the growth substratum.

Procedure:

- Pour off growth medium.
- Wash each flask with 7-8 ml PBS with EDTA for a few minutes. Repeat one time.
- Add 5 ml of trypsin to each flask. Pour off as the cells become round.
- Add 2 ml of trypsin to each flask and leave in incubator at 37 °C and 5 % CO_2 until all cells have detached.
- Add 10 ml growth medium to inhibit the trypsin.
- Transfer 2 ml of resuspended cells to each new flask with 20 ml medium.

2.5.3 Freezing cells

DMSO is added to the growth medium to prevent crystal formation in the cells, as the water freezes.

Procedure:

- Make freeze medium by adding 10 % sterile filtered DMSO to growth medium with 10 % FBS.
- Trypsinate cells according to 2.5.2.
- Centrifuge cells at 1500 x g at 4-5 °C for 5 minutes.
- Remove supernatant and resuspend cells in 1 ml freeze medium.
- Transfer to a cryovial and place in -80 °C freezer for 2 hours before transfer to liquid nitrogen.

2.5.4 Growing of MDCK cells on filter

In order to study the polarization of MDCK cells, the cells are grown on filters, where they will obtain the same polarization as *in vivo*. Epithelial cells form a single cell layer with tight junctions, which connect the cells firmly together and prevent passage of molecules and ions between the apical and basolateral sides of the cell monolayer (figure 2.1). By collecting the apical and basolateral medium separately, it is possible to study apical and basolateral secretion of molecules.

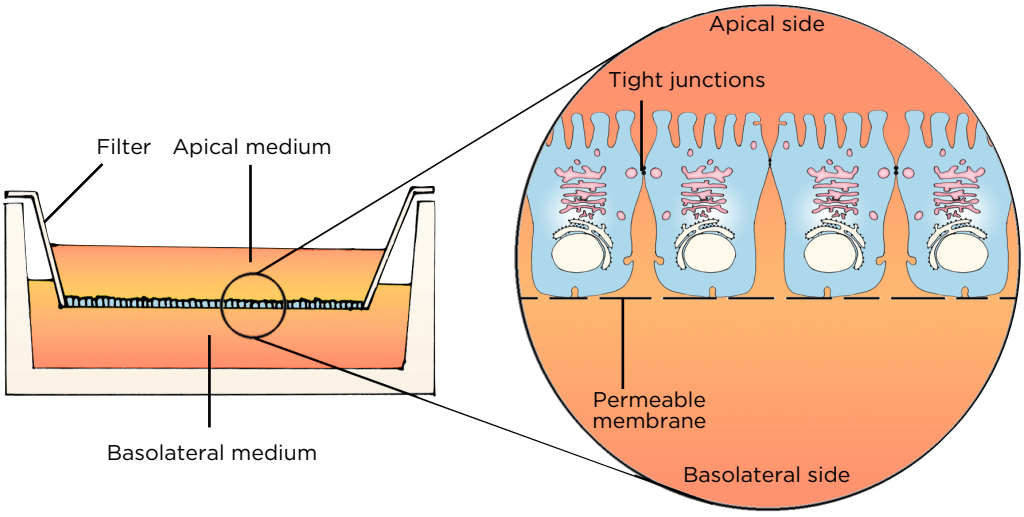

Figure 2.1 - Polarized MDCK II cells on filter
The cells are grown on a filter where they form a tight monolayer with separate apical and basolateral sides.

Procedure:

- Trypsinate cells (section 2.5.2).
- Transfer the cell suspension to a 15 ml tube and centrifuge at 1500 x g for 5 minutes.
- Remove supernatant and resuspend cells in 10 ml growth medium.
- Mount the filters on polypropylene holders and place in a 150 mm Petri dish with 90 ml medium.
- Add 1.6 ml of resuspended cells to each filter.

Transfer MDCK cells to an incubator at 37 °C and 5 % CO_2 for 3-4 days to achieve confluent monolayers. For subsequent individual treatment of each filter in experiments, transfer the filters to 6 well plate and add 1 ml growth medium apically and 2 ml basolaterally. If the samples are destined for Western blot, it is beneficial to use medium without serum.

2.5.5 Growing MDCK cells on 500 cm^2 plates

Large 500 cm^2 plates are used for subcellular fractionation and other applications where large amounts of cells are required.

Procedure:

- Add 90 ml growth medium to the plate.
- Trypsinate one cell flask (75 $cm^{2)}$ and transfer the resuspended cells (12 ml) to the plate.
- Place the cells in an incubator at 37 °C and 5 % CO_2 for 4-5 days.

2.5.6 Harvesting cells

When cells are analyzed by Western blotting, harvesting is carried out using a cellscraper instead of trypsin.

2.5.6.1 Harvesting cells from flasks

Procedure:

- Remove the growth medium.
- Wash cells with 10 ml PBS with EDTA.
- Add 5 ml PBS and use a cell scraper to detach the cells.
- Transfer the cells to a 15 ml tube and centrifuge at 1500 x g for 5 minutes.
- Remove the supernatant.
- Add 6 ml lysis buffer and incubate on ice for 30 minutes.

2.5.6.2 Harvesting cells from filters

Procedure:

- Transfer the 6 well plate with filters to ice.
- Harvest apical and basolateral media to microfuge tubes. Centrifuge the apical medium for 5 minutes at 1500 x g and transfer the supernatant to a new tube in order to remove possible loose and dead cells in the medium.
- Add 2 ml cold PBS to both sides of each filter and leave with shaking for 15 minutes before removing the PBS. Repeat one time.
- Add 2 ml lysis buffer to the apical side and leave on shaker for 30 minutes, followed by transfer of cell lysates to microfuge tubes.

2.5.7 Transfection using FuGENE 6

Transfection is a method for introducing exogenous DNA into cells. There are two main methods of transfection: stable and transient transfection. In stable transfection the plasmid is integrated into the genome of the transfected cells and the expression of the DNA-product from the plasmid is therefore also expressed in the daughter cells. In transient transfection, the plasmid is not integrated into the genome and will be diluted upon mitosis or degraded.

FuGENE® 6 (Roche) is a lipid based transfection reagent that forms a complex with DNA, which is taken up by the cell. It is suitable for both stable and transient transfection.

Procedure:

- Grow cells in cell dishes. The cells are ready for transfection when 50-70 % confluent.
- Dilute FuGENE 6 with serum-free medium:
  - Add 6 µl of FuGENE 6 directly into 93 µl of serum-free medium.
  - Mix and leave for 5 minutes at room temperature.
- Add 2 µg of DNA to the FuGENE solution. (3:1 ratio)
  - Mix and leave for 45 minutes at room temperature.
- Add FuGENE:DNA complex to the cells in a drop-wise manner.
  - Swirl and leave in incubator (37 °C and 5 % CO_2) for 24 or 72 hours for transient and stable transfection respectively.

Make a control dish by leaving out the DNA.

2.5.8 Dilution of transfected cells

For stable transfection, the cells are diluted in medium with G418, 72 hours after addition of FuGENE. G418 is an aminoglycoside antibiotic and allow selection of cells transfected with plasmids containing the gene for kanamycin resistance. The dilutions increase the chance of an optimal density of positive clones, because the efficiency of the transfection is unknown at this point.

Trypsinate cells according to 2.5.2.2 before resuspending in 9 ml of growth medium with G418. Add the following amount of resuspended cells to cell culture dishes:

Dish 1: 1:200 dilution

$$\frac{x}{14} = \frac{1}{200} \rightarrow x = \frac{14}{200} = 0.07 \text{ ml} = 70 \text{ µl}$$

70 µl of trypsinated cells are added to medium with G418 to a total volume of 14 ml.

Dish 2: 1:100 dilution

$$\frac{x}{14} = \frac{1}{100} \rightarrow x = \frac{14}{100} = 0.14 \text{ ml} = 140 \text{ µl}$$

140 µl of trypsinated cells are added to medium with G418 to a total volume of 14 ml.

Dish 3: 1:30 dilution

$$\frac{x}{14} = \frac{1}{30} \rightarrow x = \frac{14}{30} = 0.467 \text{ ml} = 467 \text{ µl}$$

467 µl of trypsinated cells are added to medium with G418 to a total volume of 14 ml.

Dish 4: Remaining cells

$$10 \text{ ml} - (467 \text{ µl} + 140 \text{ µl} + 70 \text{ µl}) \approx 9.3 \text{ ml}$$

9.3 ml of trypsinated cells are added to medium with G418 to a total volume of 14 ml.

Leave the culture dishes with the diluted cells in the incubator (37 °C and 5 % CO_2) until all the cells on the control dish are dead. The colonies containing transfected cells can be picked by removing the growth medium and placing a small metal ring over the colony. To more easily locate the colonies for placement of the metal rings, the location of the colonies can be indicated with a marker outside, on the bottom of the dish. Add 200 µl of trypsin into the metal ring and transfer the subsequently loosened cells to a glass bottomed microwell dish with 2 ml growth medium with G418, for examination of recombinant GFP-tagged protein expression in the confocal microscope. Cell lines with a high level of expression are trypsinated and transferred to a flask containing 10 ml growth medium with G418. Leave in incubator until the cells are confluent.

2.5.9 Detection of protein expression by confocal microscopy

Confocal microscopy is an imaging technique used for studying expression, localization, distribution and mobilization of fluorescent proteins and lipids. The advantage of confocal microscopy is the ability to control the depth of the focal plane by eliminating the out of focus light.

All the expressed recombinant proteins were tagged with GFP fused in the N-terminal part of the protein. GFP was originally isolated from *Aequorea victoria* and is a very commonly used reporter gene. The GFP protein exhibits green fluorescence when exposed to violet and blue light (395 and 475 nm). GFP fluorescence does not require any cofactors or substrates making it an ideal reporter for transfection.

2.5.10 Golgi apparatus staining

The Golgi marker BODIPY® TR (Invitrogen) is a fluorescent ceramide analogue. At 4 °C, the ceramide analogues are inserted into the plasma membrane. As the temperature is elevated, the lipid transport becomes more active, transporting the ceramide via small vesicles to the ER. From there, the ceramide transfer protein (CERT) transports the ceramide to the Golgi apparatus.

Procedure:

- Grow cells on glass bottomed microwell dishes.
- Wash cells with cold PBS
- Add cold 1.5 ml HBSS
- Add 5 µM BODIPY TR ceramide and transfer the cells onto ice and leave for 30 minutes.
- Remove HBSS and wash several times using ice-cold growth medium.
- Add 1.5 ml growth medium to the cells and transfer them to the incubator (37 °C and 5 % CO_2) for 30 minutes.

2.5.11 Hoechst Staining

The blue fluorescent Hoechst dyes are cell permeable, DNA binding stains that allows the visualization of the nucleus, that are extensively used in fluorescence microscopy.

Procedure:

- Add 3 µl of Hoechst dye to the cells and leave for 20-30 minutes in incubator.
- Wash several times with growth medium.

2.6 Concentration and purification of proteins

2.6.1 Concentration of proteins using a vacuum centrifuge

If the concentration of proteins in a sample is too low or the sample volume is larger than the desired volume, it is possible to reduce the volume in a vacuum centrifuge. By reducing the pressure to >1 mbar, the solvent will evaporate at room temperature.

2.6.2 Chloroform/methanol precipitation of proteins

Chloroform/methanol precipitation was used to remove sucrose and concentrate proteins from subcellular fractions.

Procedure:

- Add 600 µl methanol to a 150 µl sample. Mix thoroughly.
- Add 150 µl chloroform. Vortex for 10 seconds.
- Add 450 µl water. Vortex. Sample should appear cloudy white.
- Centrifuge at 13 000 x g for 5 minutes.
- A white disk of protein should form between the organic layer at the bottom and the aqueous layer at the top. Discard the aqueous layer.
- Add 650 µl of methanol and invert the tube three times.
- Centrifuge at 13 000 x g for 5 minutes.
- Remove all liquid and allow pellet to air dry before resuspending the proteins in the desired volume.

2.6.3 Sephadex™ G-50 Fine gelfiltration

Sephadex G-50 Fine (GE Healthcare) separates proteins with a molecular weight between 1000 and 30 000 Da. Macromolecules with a mass larger than 30 000 Da will be eluted in V_0, while smaller molecules will remain in the column. Radioactive molecules not incorporated into macromolecules will be removed by G-50 Fine gelfiltration.

Procedure:

- G50 Fine needs to be swelled in 10 ml dH_2O/g for at least 3 hours, before use.
- Cut 10 ml pipettes at the 7 ml mark and insert a small piece of glass wool into the tip of the pipette.
- Add 4 ml of swelled G-50 Fine into the pipette.
- Add 1 ml of the sample to the column with G50 Fine, and allow it to enter the gel completely.
- Elute macromolecules using 1.5 ml elution buffer or water.
- Eluate can be analyzed by scintillation counting, SDS-PAGE, immunoprecipitation (IP), or gel filtration.

2.7 Protein analysis

2.7.1 Radioactive labeling of molecules

Labeling cells with radioactive isotopes allows studies of synthesis, sorting and modification of proteoglycans (PGs) and glycoproteins.

[^{35}S]-sulfate labeling: GAG chains on proteoglycans are modified with sulfate. By replacing non-radioactive sulfate with radioactive sulfate in the medium, it is possible to determine the amount of sulfated proteoglycans synthesized during the labeling period.

[^{3}H]-glucosamine labeling: Radioactive glucosamine is incorporated into GAG chains and N-glycans. The amount of sugars attached to PGs and glycoproteins secreted into the apical and basolateral media as well as the amount remaining in the cell fraction can be determined.

Procedure:

- Grow cells in wells or on filters.
- Remove the medium from the cells.
- Use RPMI-1640 medium with 2 % FBS, without sulfate for [^{35}S]-sulfate labeling and without glucose for ^{3}H-Glucosamine labeling.
- Add the radioactive isotope to the medium with a final concentration of 0.2 mCi/ml medium.
- Add 1 ml of the radioactive medium to the wells, or when growing cells on filter, add 2 ml radioactive medium basolaterally and 1 ml RPMI-1640 without radioactive isotope apically.
- Place the cells in an incubator at 37 °C and 5% CO_2 for 20-24 hours.
- Harvest the media and centrifuge at 5000 x g for 5 minutes. Transfer supernatant to new tubes.
- Wash the cell layer with 2.0 ml PBS for 20 minutes on ice. Add PBS to both apical and basolateral sides when using cells on filter. Repeat one time.
- Remove PBS and add 1 ml lysis buffer to the cells. After 30 minutes on ice, transfer the lysis solution to tubes.

2.7.2 Liquid scintillation counting

Liquid scintillation counting is a method for detecting β-radiation. β-particles emitted from the sample will excite solvent molecules. The energy of the excited solvent is emitted as UV light to fluors in the solution. The fluor molecule will then release its excitation energy as light. These flashes of lights are counted by the scintillation counter and reported as counts per minute (CPM).

Procedure:

- Transfer 50 µl G50 Fine eluate of radioactive sample to a scintillation vial.
- Add 3 ml scintillation cocktail (Ultima Gold™ XR, Perkin Elmer) and mix well.
- Place vials in scintillation counter and select appropriate program.

2.7.3 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a method for separating proteins according to their molecular mass. SDS is an anionic detergent. By boiling the samples with SDS, the proteins are denatured due to disruption of non-covalent bonds and SDS bound to the proteins. Approximately one SDS anion for every two amino acids. Since all the protein molecules are negatively charged, induced by the binding of SDS, leaving only the molecular mass as the determining factor of each protein's mobility in the gel. Small proteins will move faster than large ones. The concentration of acrylamide will also have an effect on the mobility of the proteins. A protein will move more slowly in a gel with a high percentage of acrylamide than in a gel with a lower percentage.

Procedure:

- 20 µl of the sample is mixed with 7.5 µl 4X XT Sample Buffer (Bio-Rad) and 1.5 µl 20X XT Reducing Agent (Bio-Rad).
- Boil the samples at 96 °C for 5 minutes.
- Centrifuge the samples at 3000 x g for 5 minutes.
- Apply the samples on the gel.
- Run at 180 V and 90 mA per gel for 1 hour and 15 minutes.
- After electrophoresis, transfer the gel to fixing solution for 40 minutes.
- Transfer gel to Amplify™ solution (GE Healthcare), which amplifies the radioactive signal, thereby reducing the time needed for exposure.
- Dry gel at 70 °C for 1 hour and 30 minutes.

Gels with [^{35}S]-sulfate labeled samples are placed in a cassette together with a Molecular Dynamics LE Storage Phosphor screen over night, before the screen is scanned using a Typhoon™ 9400 Variable Mode Imager (GE Healthcare). Gels with ^{3}H-Glucosamine labeled samples are placed in a cassette together with a film (Amersham™ Hyperfilm™ MP, GE Healthcare) and left at -80 °C for 1-3 weeks depending on the amount of radioactivity. The various protein bands can be quantified using ImageQuant™ TL (GE Healthcare).

2.7.4 Western blotting

Western blotting, or immunoblotting, is a method for detecting specific proteins in a sample. The proteins are first separated by electrophoresis (e.g. SDS-PAGE), before transfer to a membrane (nitrocellulose or PVDF) by electroblotting, where the proteins are detected by using antibodies specific for the target proteins.

Because the membrane has the ability to bind all proteins the membrane has to be blocked before detection with antibodies, otherwise the antibodies will bind to the membrane in a non-specific manner. By leaving the membrane in a blocking solution with non-fat dry milk, the proteins in the blocking solution will bind to all parts of the membrane not already occupied by the proteins transferred from the gel. Thus, when adding the antibody the only place site to bind is the specific epitope binding site on the target protein.

The antibodies used in this thesis are:

- Primary antibody: GFP antibody (ab6556) (Abcam)
- Secondary antibody: Anti-rabbit IgG, alkaline phosphatase linked antibody (GE Healthcare)

Procedure:

- Prepare the following:
  - Two pieces of blotting paper in cathode buffer.
  - Two pieces of blotting paper in anode buffer.
  - Activate Hybond™-P PVDF membrane (GE Healthcare) in methanol for 10 seconds before transferring to anode buffer.
- After electrophoresis leave gel in cathode buffer for 5 minutes.
- Assemble blotting sandwich on Trans-Blot® Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) in the following order from bottom and up:
  - Blotting paper with anode buffer.
  - PVDF membrane.
  - The gel.
  - Blotting paper with cathode buffer
- Blot at 25 V and 170 mA for 1 hour.
- Transfer the membrane to TTBS with 5 % dry milk for 1 hour with tilting.
- Incubate the membrane with primary antibody (1:10 000) in TTBS with 1 % dry milk overnight at 4 °C with tilting.
- Wash the membrane 3 x 15 minutes with TTBS.
- Incubate the membrane with secondary antibody (1:10 000) in TTBS with 1 % dry milk for 1-2 hours at room temperature with tilting.

- Wash the membrane 3 x 15 minutes with TTBS, followed by 1 x 15 minutes with TBS.
- Develop membrane by adding 1.5-2 ml ECF™ substrate (GE Healthcare) to the membrane for 5 minutes. In the presence of alkaline phosphatase, a phosphate group is cleaved from the substrate; creating a fluorescent product. Dry the membrane and scan using the Typhoon 9400 Variable Mode Imager.

2.7.5 Subcellular fractionation

Density gradient subcellular fractionation is a method for separating cellular organelles in a density gradient according to their density. When homogenizing the cells, the plasma membrane is destroyed, while most of the organelles remain intact. After removing the nucleus by centrifugation, the postnuclear supernatant (PNS) is applied onto a sucrose gradient. During centrifugation, the organelles will move to the area of the gradient that has the same density as the organelles themselves. This will result in a concentration of different organelles in different regions of the gradient.

The Golgi fraction can be used for in vitro GAG synthesis studies.

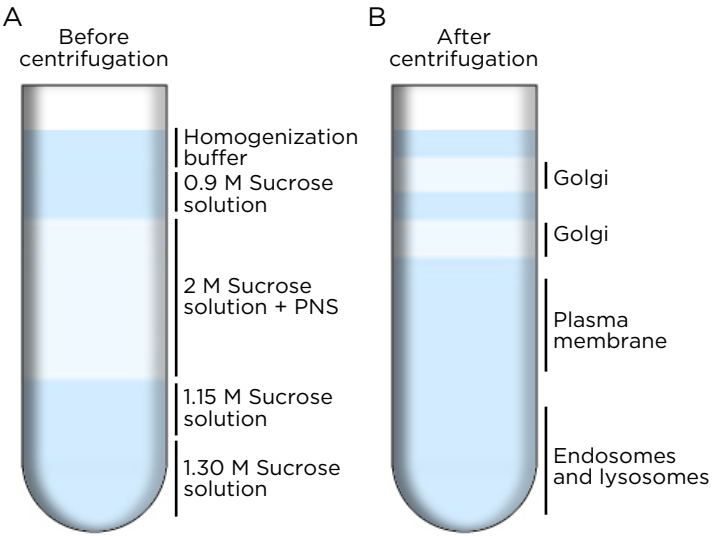

Figure 2.2 - Subcellular fractioning
(A) The setup of a sucrose gradient. (B) The Golgi fractions appear as two white bands in the upper part of the gradient.

Procedure:

- Grow cells on large, 500 cm^2, plates (2.5.5)
- Transfer plate onto ice for 10 minutes.
- Remove growth medium and wash with 15 ml homogenization buffer.
- Add 15 ml homogenization buffer and detach the cells with a cell scraper. Transfer cell solution to a 50 ml tube. Repeat one time.
- Centrifuge cells at 1500 x g for 5 minutes at 4 °C.
- Remove supernatant and resuspend the cells in homogenization buffer to a total volume of 5 ml.

- Homogenize the cells by forcefully sucking up the resuspended cells using a syringe with a 22 ½ G needle bent into an S-shape. Repeat ten times.
- Transfer cell homogenate to a 15 ml tube.
- Centrifuge tube at 2500 x g for 10 minutes at 4 °C.
- Transfer the supernatant to a new 15 ml tube. This is the postnuclear supernatant (PNS).
- Make a sucrose gradient in a Beckman Ultra-Clear™ (1 x 3 ½ inches) centrifuge tube. Pipette each layer carefully to avoid mixing:
  1. 5 ml 1.30 M sucrose solution.
  2. 5 ml 1.15 M sucrose solution.
  3. Mix 8.4 ml of PNS (Adjust volume with homogenization buffer if necessary) with 6.6 ml 2 M sucrose solution, to a total of 15 ml.
  4. 6 ml 0.9 M sucrose solution.
  5. 6 ml homogenization buffer.
- Centrifuge the gradients at 28 000 x g for 4 hours and 30 minutes in a SW32 rotor. N.B! make sure opposite tubes are balanced within 0.01 g of each other.
- Harvest the gradient by careful and sequential pipetting of 2 ml fractions from the top. The Golgi fractions can be observed as two pale, white bands in the upper half of the gradient (figure 3.2B).

2.7.6 *In vitro* GAG synthesis

Use the upper Golgi fraction from the subcellular fractionation. This is the fraction with the least contamination of non-Golgi components.

Procedure:

- For each sample, mix the following in a tube:
  - 250 µl Golgi fraction.
  - 250 µl cytosol.
  - 50 µl ATP regenerating system:
    - 1 mM ATP.
    - 10 mM phosphocreatine.
    - 0.5 mM UTP.
    - 50 µg creatine phosphokinase.
  - 5 mM Mg^{2+}.
- Add 0.2 mCi [^{35}S]-sulfate to each sample.
- Incubate for 2 hours at 37 °C in a water bath.
- Transfer samples to ice and dilute to 1 ml with water.
- Purify macromolecules on G-50 Fine columns using scintillation counting and SDS-PAGE analysis.

2.7.7 Chondroitinase ABC treatment

Chondroitinase ABC (cABC) treatment is a method for degradation of chondroitin sulfate (CS) and dermatan sulfate (DS) GAG chains. cABC is an enzyme that cleaves both CS and DS chains into tetra- and disaccharides.

Procedure:

- Dry sample using the vacuum centrifuge (2.6.1).
- Resuspend sample with 20 µl cABC buffer.
- Add 10 mU cABC enzyme.
- Leave at 37 °C for 1 hour.
- Stop reaction by boiling sample at 96 °C for 2 min.

2.7.8 Heparinase treatment

Heparinase is an enzyme that selectively cleaves heparan sulfate and can therefore be used as a method for removal of heparan sulfate (HS) GAG chains.

Procedure:

- Add the following to a 15 µl sample:
  - 15 µl 2X buffer.
  - 1 µl Heparinase.
- Leave at 28 °C ON.

2.7.9 PNGase F treatment

Peptide: N-Glycosidase F (PNGase F) is an enzyme that removes N-glycan chains from glycoproteins. It cleaves the bond between an asparagine in the protein and the first N-acetyl glucosamine of the glycan chain. The method is used for detection of N-glycans on proteins.

Procedure:

- Mix 22 µl of the sample with 2.4 µl denaturing buffer.
- Leave at 96 °C for 10 min.
- Add:
  - 3 µl G7 buffer.
  - 3 µl NP40.
  - 1 µl PNGase F.
- Leave at 37 °C for 1 hour.

3. RESULTS

The work carried out in this thesis can be divided into two parts: Work with MDCK cells and work with LNCaP cells. The experiments with MDCK cells constitute the majority of the work performed and can again be divided into two parts, analysis of STAMP2 and analysis of STAMP2 transfected cells.

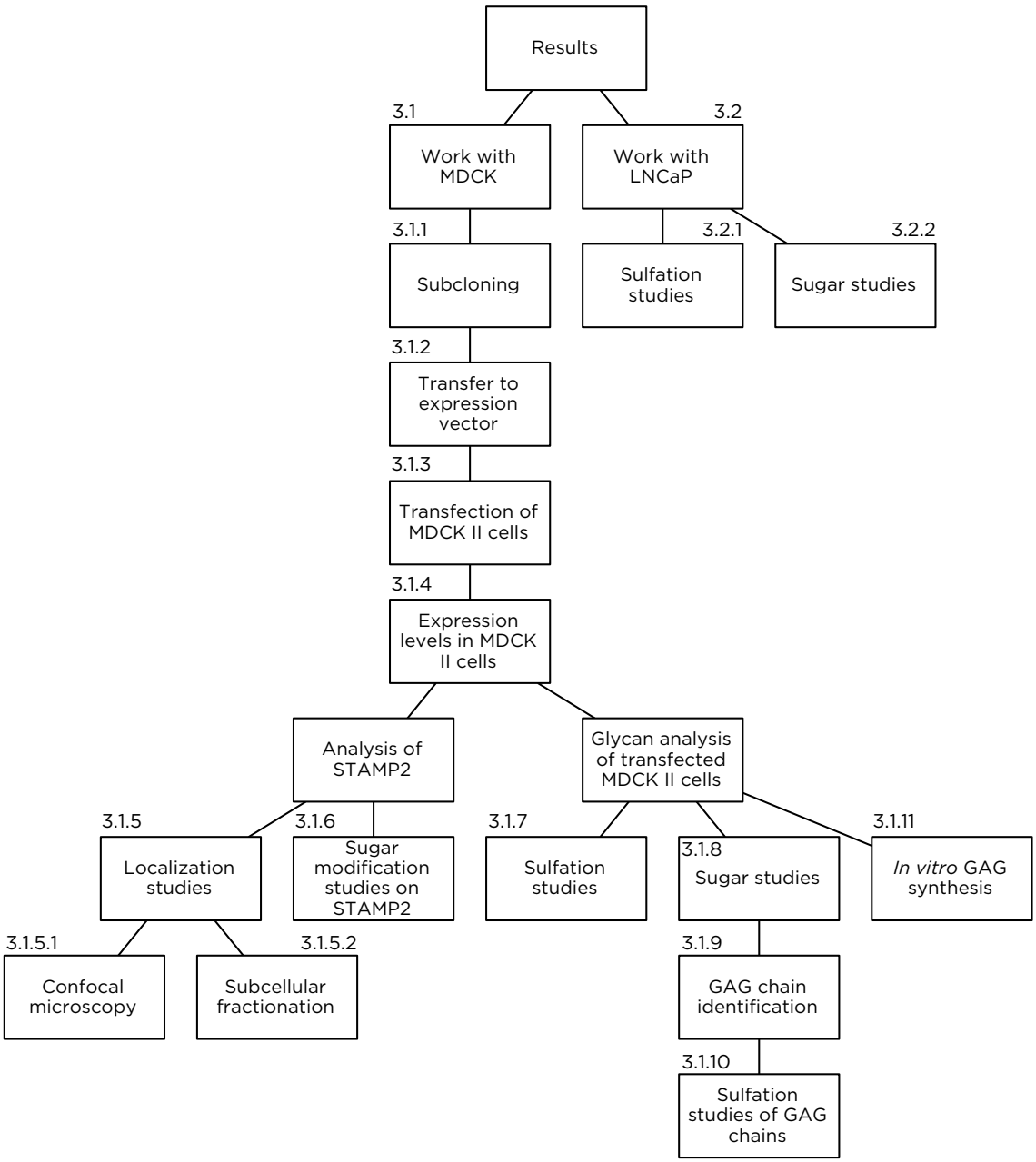

Figure 3.1 - Overview of work performed

3.1 Work with MDCK cells

A construct with GFP N-terminally attached to STAMP2, called GFP-STAMP2 (GFP-S2) had previously been made by another group (Korkmaz et al. 2005). This group also constructed a deletion mutant of STAMP2, S2', with a nine nucleotide deletion in the ferric-reductase domain (ORD) in the C-terminal region. Both served as a basis for the production of two new constructs, STAMP2-GFP (S2-GFP) and STAMP2'-GFP (S2'-GFP), both having the GFP tag moved 20 amino acids downstream from the start (ATG) of STAMP2. S2'-GFP also have a 9 nucleotide deletion in the ORD (figure 3.2). Moving GFP away from the N-terminal could expose a possible sorting signal, that would otherwise be blocked, while the deletion of amino acids in the ORD could knock out or change the function of this domain. GFP-S2, S2-GFP and S2'-GFP were all stably transfected into MDCK cells, for investigation of subcellular localization of the STAMP2 variants, as well as synthesis and sorting of glycoproteins and proteoglycans.

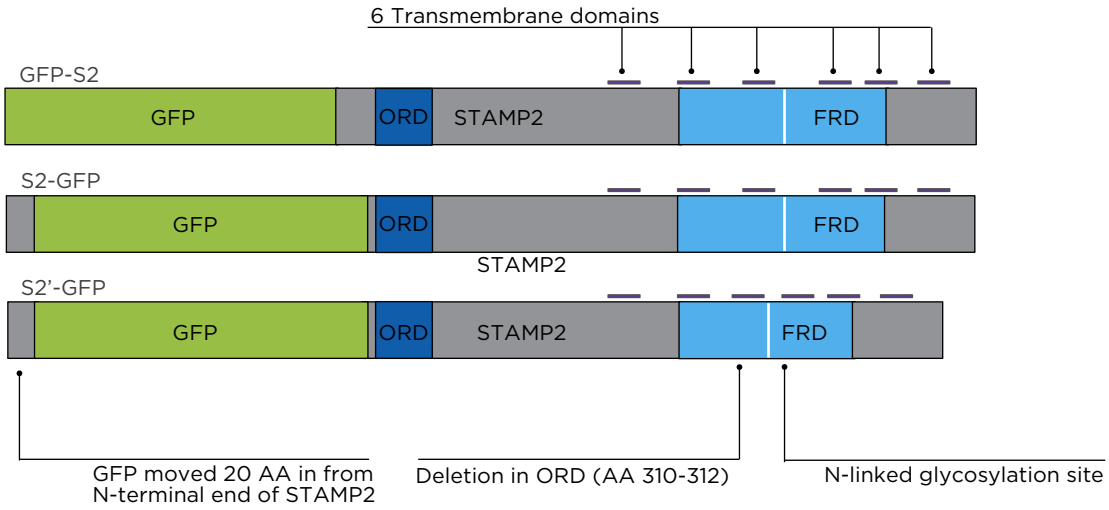

Figure 3.2 - Overview of the STAMP2 constructs.
Green Fluorescent Protein (GFP) is indicated in green, flavin-NAD(P)H binding oxidoreductase domain domain in dark blue and the ferric-reductase domain (FRD) in light blue. The six transmembrane regions are indicated by grey lines above each construct. The predicted N-linked glycosylation site is indicated by a white line.

3.1.1 Subcloning of S2-GFP and S2'-GFP

An overview of the subcloning and molecular cloning process performed is shown in figure 3.3. A more detailed overview of the PCR steps is shown in figure 3.4.

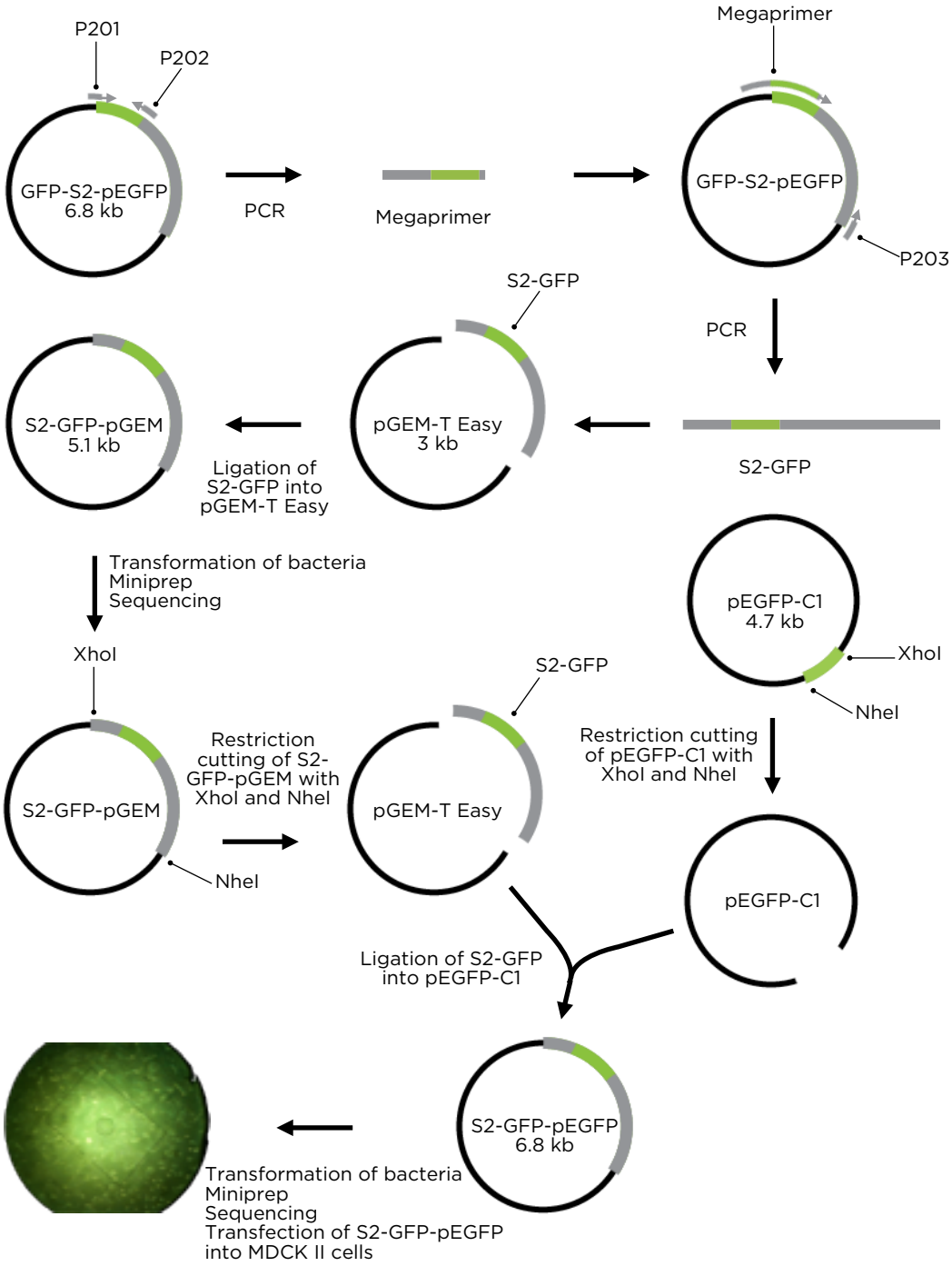

Figure 3.3 - Strategy for subcloning of STAMP2 constructs

A megaprimer was amplified by PCR using P201 and P202 (see appendix 1 for primer sequence) with GFP-S2-pEGFP-C1 as template. The megaprimer was then used to amplify GFP-S2 together with P203 (see appendix 1 for primer sequence). The new construct, S2-GFP was ligated into pEGEM-T Easy and sequenced. Plasmids with correct insert were digested with XhoI and NheI, and the fragment was ligated into pEGFP-C1 and sequenced. Plasmids containing the clone with correct sequence were used in stable transfection of MDCK II cells. Same procedure was used for S2'-GFP, except the use of the deletion mutant, S2', in a pcDNA vector as template in the second PCR reaction.

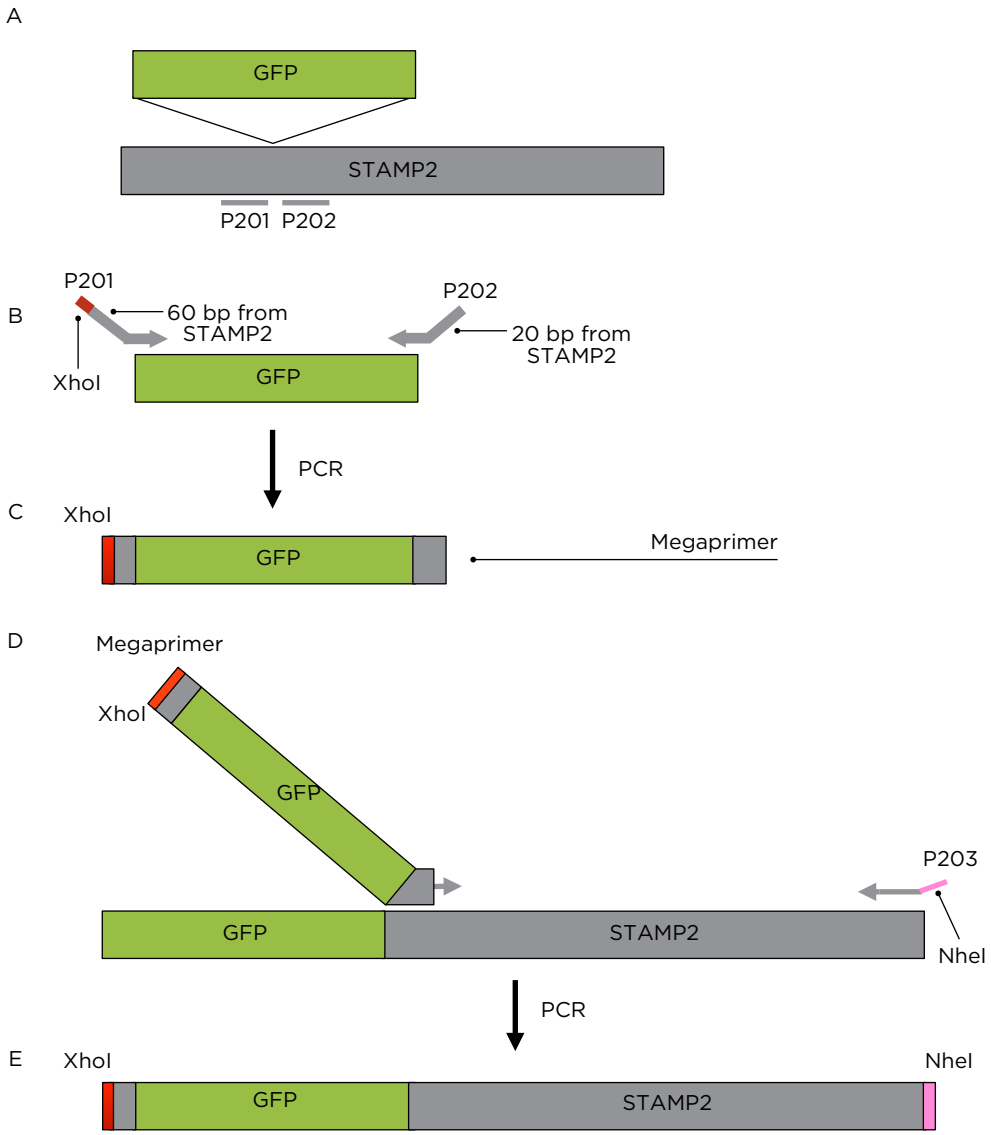

Figure 3.4 - Subcloning of S2-GFP
(A)The purpose of the subcloning is to insert GFP 20 amino acids downstream from the N-terminal start codon of STAMP2. (B) GFP is amplified using two primers containing parts of STAMP2 sequence. The forward, P201, contains a XhoI restriction site at its N-terminal end. (C) The PCR product is used as a megaprimer in (D) together with a reverse primer, P203, containing a NheI restriction site.

A megaprimer containing GFP was amplified by PCR using primers P201 and P202 (For primer sequences, see appendix 1) containing the restriction sites XheI and NheI respectively. The PCR was performed as described in section 2.3.1. Due to the differences in melting temperatures for the two primers, four samples were tested at four different temperatures for the annealing step: 57.5 °C, 59.9 °C, 63.7 °C and 65 °C. A fraction of each of the PCR products was applied on a 0.8 % agarose gel (Figure 3.5). The PCR product was amplified at all temperatures, and the size corresponds to the theoretical size (864 bp). The PCR product was isolated using QIAquick Gel Extraction kit from QIAGEN (Section 2.2.4).

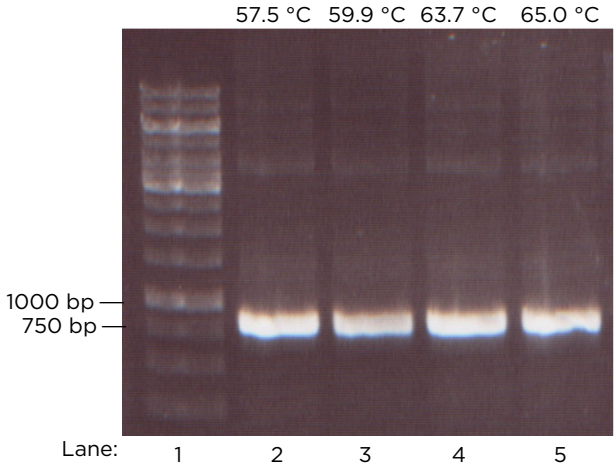

Figure 3.5 - PCR amplification of GFP. GFP was amplified by PCR using primers P201 and P202, to create a megaprimer, at four different annealing temperatures. A fraction of each sample was applied on a 0.8 % agarose gel.

The isolated fragment was used as a megaprimer for the second PCR, together with P203, a primer containing the C-terminal portion of STAMP2 with a XhoI restriction site (Figure 3.4d). Two concentrations, 5 pmol and 1 pmol, of the megaprimer were used to amplify both the GFP-S2 and S2' DNA products. The PCR fragments were applied on a 0.8% agarose gel (figure 3.6) and the PCR products corresponding to the correct size (as indicated by asterisks) in lane 2-7 were purified using QIAquick Gel Extraction kit from QIAGEN.

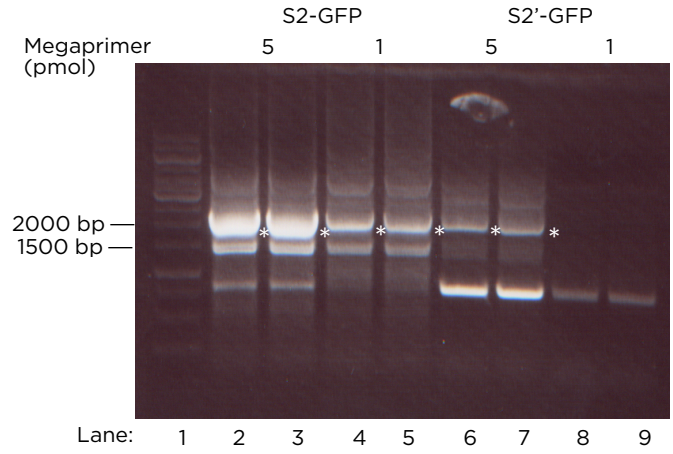

Figure 3.6 - The S2-GFP and S2'-GFP PCR products GFP-S2 and S2' were amplified by PCR using two different concentrations of megaprimer (5 or 1 pmol). The samples were applied on a 0.8 % agarose gel, and the isolated PCR products are indicated by asterisks (*).

The purified S2-GFP and S2'-GFP PCR fragments were ligated into the cloning vector pGEM-T Easy (S2-GFP-pGEM and S2'-GFP-pGEM), before transformation into competent *E. coli* cells (section 2.1.4). The transformed bacteria were plated on agar plates with ampicillin. Bacteria colonies were selected from the agar plates and grown ON in 5 ml LB medium with ampicillin. QuickCheck was performed to verify the presence of plasmids with inserted PCR product in the transformed bacteria (figure 3.7). The plasmids indicated by asterisks (fig. 3.7A, lane 2 and 3, fig. 3.7B, lane 2, 3, 5, and 7) were isolated, and an analysis using EcoRI (section 2.3.2) was performed (figure 3.8).

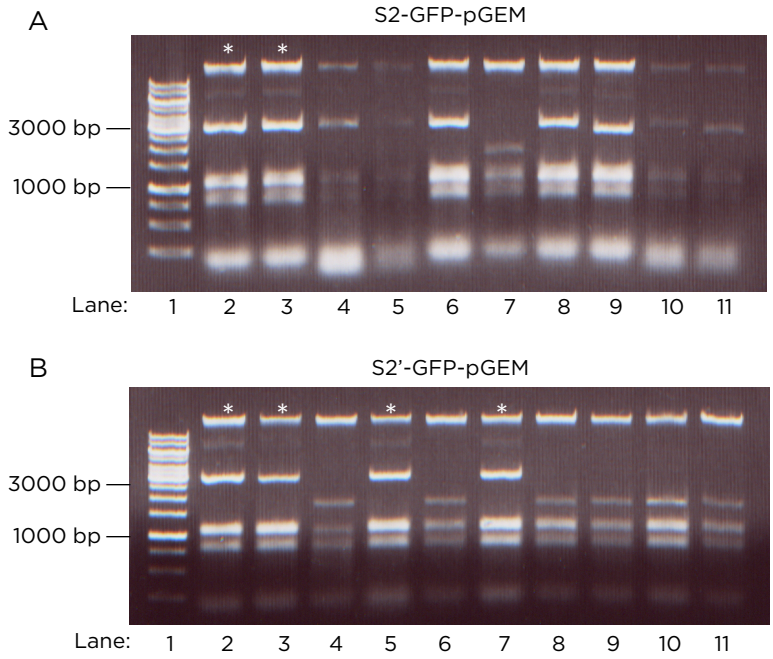

Figure 3.7 - QuickCheck of S2-GFP-pGEM and S2'-GFP-pGEM.
Bacteria culture was treated with phenol and chloroform. The aqueous phase, containing DNA, was loaded on a 0.8 % agarose gel. Samples with plasmid of correct size indicated by asterisks (*).

The restriction analysis resulted in three fragments: One fragment larger than 2000 bp (▪), (corresponding to the size of the S2-GFP fragment (2077 bp) or S2'-GFP fragment (2068 bp)), one at 3000 (◆)(corresponding to the size of the pGEM-T Easy vector (2997 bp)) and one around 5000 bp (●)(corresponding to a linear S2-GFP-pGEM or S2'-GFP-pGEM). A fourth theoretical fragment, 59 bp long, was not observed in the gel.

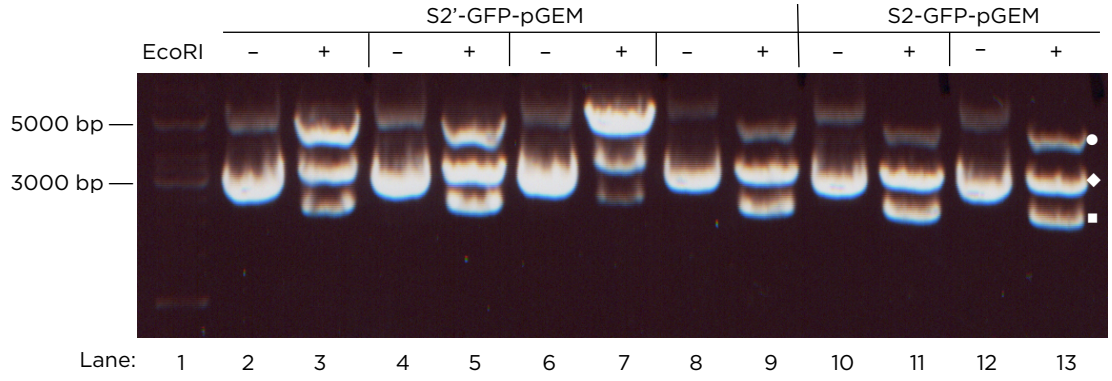

Figure 3.8 - Restriction analysis of S2-GFP-pGEM and S2'-GFP-pGEM.
S2-GFP-pGEM and S2'-GFP-pGEM were treated with the restriction enzyme EcoRI and loaded on a 0.8 % agarose gel.

All the four S2'-GFP-pGEM and the two S2-GFP plasmids had DNA insert with a correct size, and were sent to the ABI lab at Department of Molecular Biosciences/Department of Biology for sequencing (section 2.4).

3.1.2 Transfer of STAMP2-GFP and STAMP2'-GFP to the expression vector pEGFP-C1

To transfer S2-GFP and S2'-GFP from the cloning vector to the expression vector, both S2-GFP-pGEM and S2'-GFP-pGEM, and the expression vector, pEGFP-C1 (see appendix 1), were digested with NheI and XhoI restriction enzymes, prior to analysis on agarose gels (Figure 3.9).

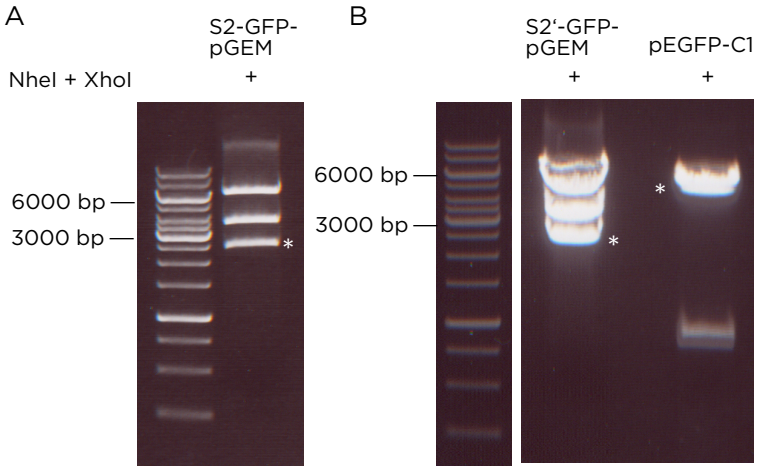

Figure 3.9 - S2-GFP-pGEM, S2'-GFP-pGEM and pEGFP-C1 digested with NheI and XhoI. (A) S2-GFP-pGEM cut with NheI and Xhol and loaded on a 0.8 % agarose gel. (B) S2'-GFP-pGEM (left) and pEGFP-C1 (right) cut with NheI and XhoI and applied on a 0.8 % agarose gel. Isolated and purified DNA fragments are indicated by asterisks (*).

The DNA fragments corresponding to S2-GFP, S2'-GFP and expression vector pGEFP-C1 without GFP (as indicated by asterisks on figure 3.9) were isolated and purified. S2-GFP and S2'-GFP were ligated into the pEGFP-C1 vector. The plasmids, S2-GFP-pEGFP and S2'-GFP-pEGFP, were then transformed into *E. coli* cells and the bacteria were plated on agar dishes with kanamycin. Bacteria colonies were selected and Quick Check was performed to identify transformants with plasmids of correct size. As seen in figure 3.10, the bacteria contained plasmids of two different sizes.

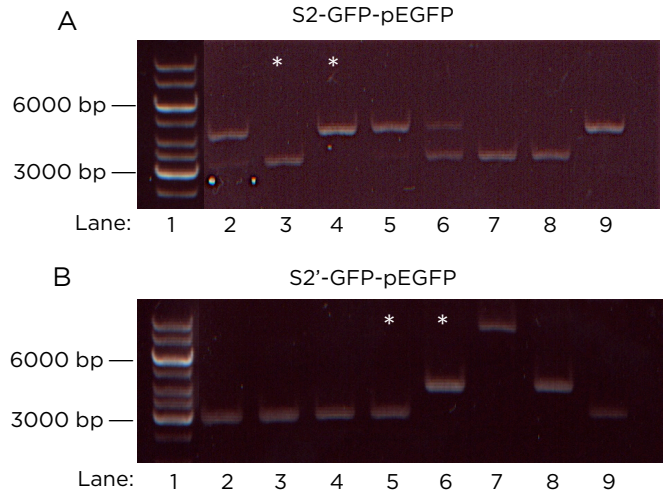

Figure 3.10 - QuickCheck of S2-GFP-pEGFP and S2'-GFP-pEGFP. Bacteria cultures were treated with phenol and chloroform and the aqueous phase containing plasmids was applied on a 0.8 % agarose gel. Plasmids selected for restriction analysis indicated by asterisks (*)

Two different plasmids were further purified. S2-GFP-pEGFP (figure 3.10A, lane 3 and 4), and the S2'-GFP-pEGFP (figure 3.10B, lane 5 and 6), were isolated and digested with NheI and XhoI, followed by agarose gel analysis. Plasmids with correct size will give two DNA molecules after the restriction analysis, 2.1 kb (the PCR product) and 4 kb (pEGFP without GFP). As seen in figure 3.11, the plasmid in lane 7 (corresponding to the plasmid seen in figure 3.10A, lane 4) and 11 (corresponding to the plasmid seen in figure 3.10B, lane 6) had DNA molecules with corresponding sizes, in addition to a larger DNA fragment of about 6 kb, corresponding to a correct linearized plasmid.

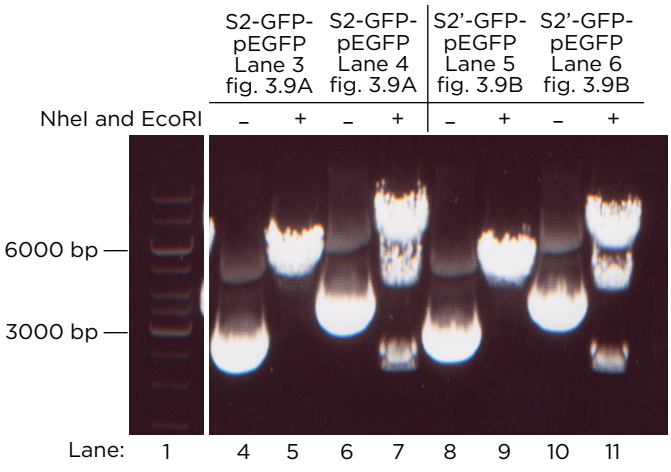

Figure 3.11 - Restriction analysis of S2-GFP-pEGFP and S2'-GFP-pEGFP. Purified plasmids were digested with NheI and XhoI, followed by application on a 0.8 % agarose gel.

S2-GFP-pEGFP and S2'-GFP-pEGFP from lane 4 in figure 3.10A and lane 6 in figure 3.10B were sent to in-house sequencing.

3.1.3 Transfection of STAMP2 variants in MDCK II cells

MDCK II cells were stably transfected with GFP-S2-pEGFP, S2-GFP-pEGFP, and S2'-GFP-pEGFP using FuGENE 6 (section 2.5.7). Cell colonies that survived the selection with G418 were examined using confocal microscopy, and only a limited number of the selected colonies

expressed the STAMP2 variants at a detectable level, and these were further studied (data not shown).

3.1.4 STAMP2 expression in MDCK II

In order to verify the expression level of the STAMP2 variants, the cell lines were grown in 75 cm^2 cell flasks and harvested (section 2.5.6.1). The cells were lysed using lysis buffer, before aliquots of cell lysate were applied onto an SDS-PAGE and Western blotted (figure 3.12). Anti-GFP antibodies were used due to the lack of effective antibodies against STAMP2.

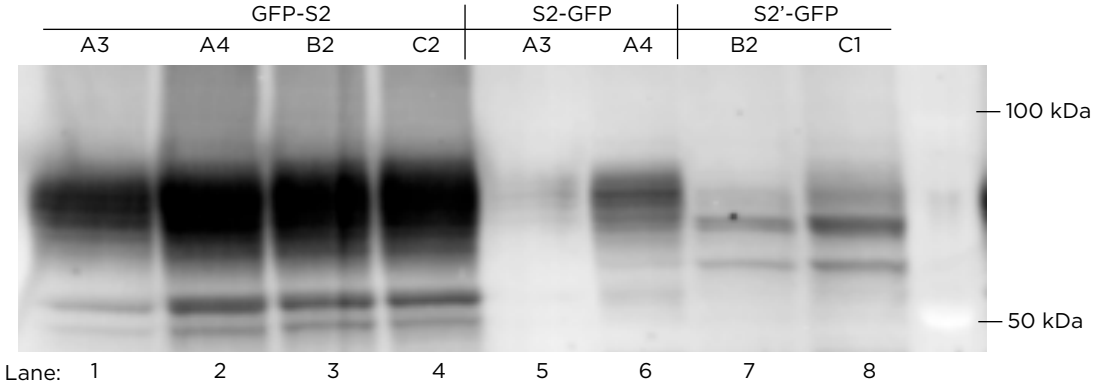

Figure 3.12 - Expression levels of GFP-S2, S2-GFP and S2'-GFP in MDCK II cells.
MDCK II cells stably transfected to express GFP-S2, S2-GFP and S2'-GFP were grown in cell flasks. The cells were harvested and lysed. 20 µl of the cell lysate was applied on a 4-12% SDS-PAGE gel and Western blotted with anti-GFP.

All four cell lines expressing GFP-S2 had a high level of expression, while for S2-GFP only one of the cell lines expressed a detectable level of STAMP2. For S2'-GFP, both cell lines had a low, but detectable level of the STAMP2 construct.

Analysis of STAMP2

Three different STAMP2 variants had been stably expressed in MDCK II cells, and we wanted to investigate the cellular localization of the expressed protein variants, as well as possible post-translational modifications of the protein.

3.1.5 Localization studies of STAMP2 in MDCK II cells

GFP-STAMP2 with GFP at the N-terminal end, might have a blocked N-terminal sorting signal. Earlier studies of GFP-STAMP2 in LNCaP cells have shown its localization to the Golgi apparatus, the plasma membrane and also to vesiculotubular structures (Korkmaz et al. 2005). It was therefore of interest to investigate whether the altered location of GFP in the protein changed the cellular localization of STAMP2.

3.1.5.1 Subcellular localization studies using confocal microscopy

In addition to evaluation of the expression level of the STAMP2 proteins, the magnification and resolution of confocal microscopes enables localization studies of proteins in cells. The localization can easily be addressed by labeling cells with markers for organelles, such as the Golgi apparatus or the nucleus, and subsequently observing the extent of co-localization with the novel protein construct. The cells were grown in glass bottomed microwell dishes over night, and Golgi staining was performed as described in section 2.5.10.

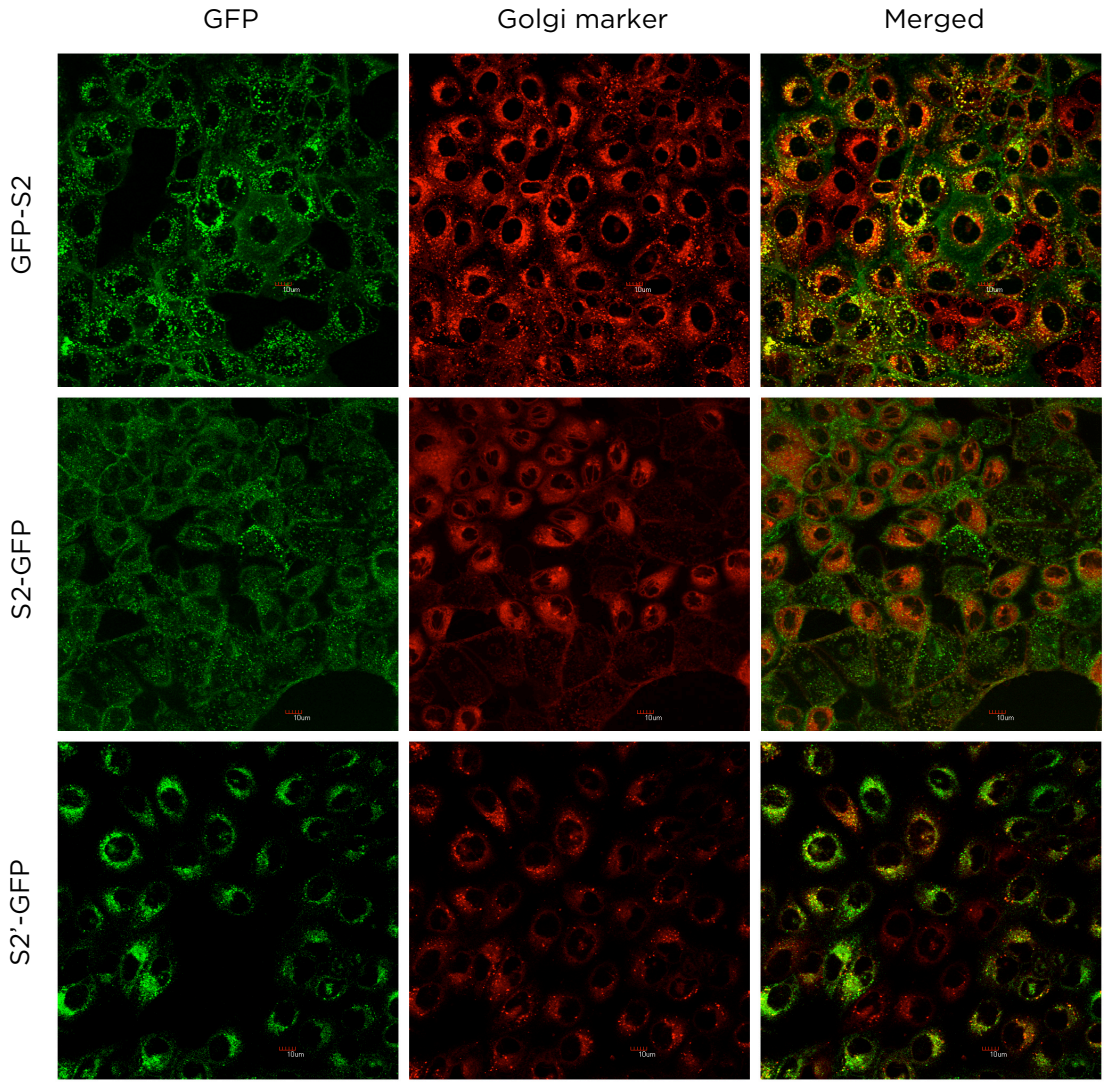

Figure 3.13 - Localization of GFP-STAMP2, STAMP2-GFP and STAMP2'-GFP in MDCK II cells.

MDCK II cells stably expressing GFP-STAMP2, STAMP2-GFP, and STAMP2'-GFP were grown in glass bottomed microwell dishes. A red fluorescent lipid Golgi marker (BODIPY TR ceramide) was added, and the proteins and organelles were visualized in a confocal microscope (Olympus IX81).

As seen in figure 3.13 there is a distinct difference in the localization of the different STAMP2 variants. While GFP-S2 showed a high degree of co-localization with the Golgi marker, S2-

GFP had a more defined localization to the plasma membrane and vesicular structures, with very little co-localization with the Golgi marker. The expression of S2'-GFP was low (figure 3.12, lane 8), but showed a perinuclear localization with some degree of co-localization with the Golgi marker, but less than for GFP-S2. The perinuclear region of the cells harbors also the ERGIC and late endosomal compartments.

3.1.5.2 Localization studies with subcellular fractionation

To further investigate the differences in localization of GFP-S2, S2-GFP, and S2'-GFP in transfected cells, subcellular localization of the STAMP2 variants by subcellular fractionation in a sucrose gradient were performed. The cells were grown on large 500 cm^2 plates, removed by scraping and homogenized, before the nuclei were removed from the cell lysates by centrifugation. The post-nuclear supernatants were applied onto sucrose gradients in order to separate the organelles according to their density (section 2.7.5). Samples from all fractions were analyzed by Western blotting with an anti-GFP antibody (figure 3.14).

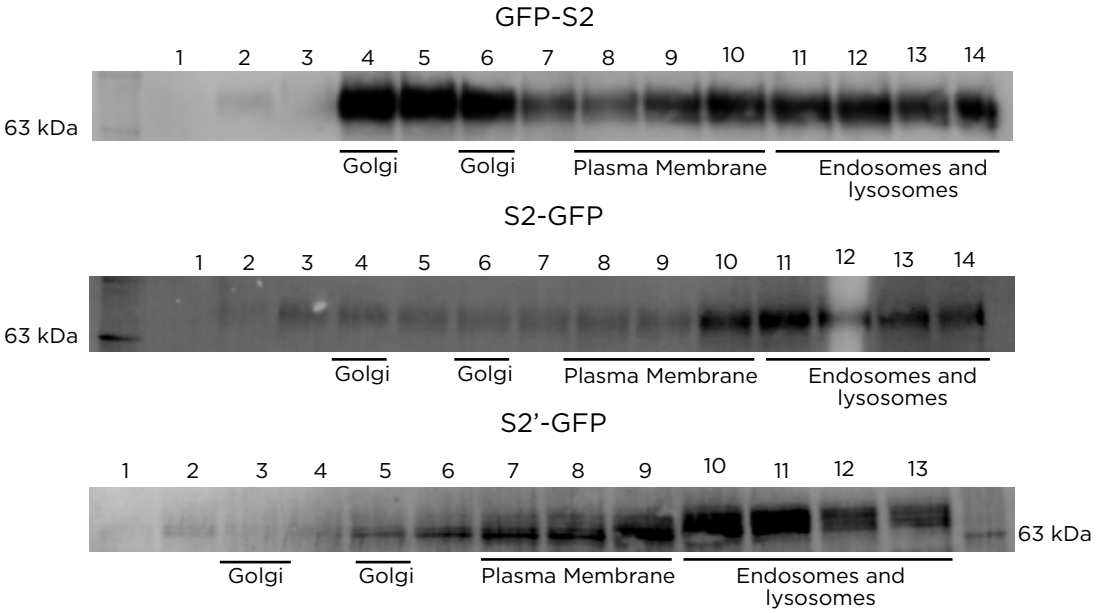

Figure 3.14 - Subcellular fractionation of GFP-S2, S2-GFP and S2'-GFP in MDCK cells
MDCK II cells stably expressing GFP-S2, S2-GFP and S2'-GFP were grown on large plates. Cells were harvested and post-nuclear supernatants were separated on sucrose gradients. Fractions were collected from the top of the gradient and aliquots were applied on 4-12 % SDS-PAGE gels and Western blotted with anti-GFP.

Fraction 4 (3 for S2'-GFP) is the fraction with the highest concentration of Golgi markers. The plasma membrane is primarily localized in fractions 8 to 10 (7-9 for S2'-GFP), while endosomes and lysosomes are more concentrated in the last fractions. The subcellular fractionation shows a clear difference in the localization of the STAMP2 variants. The results partially support what was observed in the confocal microscope. GFP-S2 is highly localized in the Golgi area, but also

appears to be at the plasma membrane and in endosome like structures. S2-GFP and S2'-GFP are localized partially at the plasma membrane and primarily in vesiculotubular membranes, most likely endosomes.

3.1.6 Sugar modification studies of STAMP2 variants

STAMP2 has one predicted N-glycan site (Chen et al. 2009). To investigate whether the STAMP2 variants were glycosylated, the cell lysates of cells expressing GFP-S2, S2-GFP, and S2'-GFP were treated with PNGase F, an enzyme that removes N-glycans from glycoproteins (section 2.7.9). The samples were applied onto SDS-PAGE gels and Western blotted (figure 3.15).

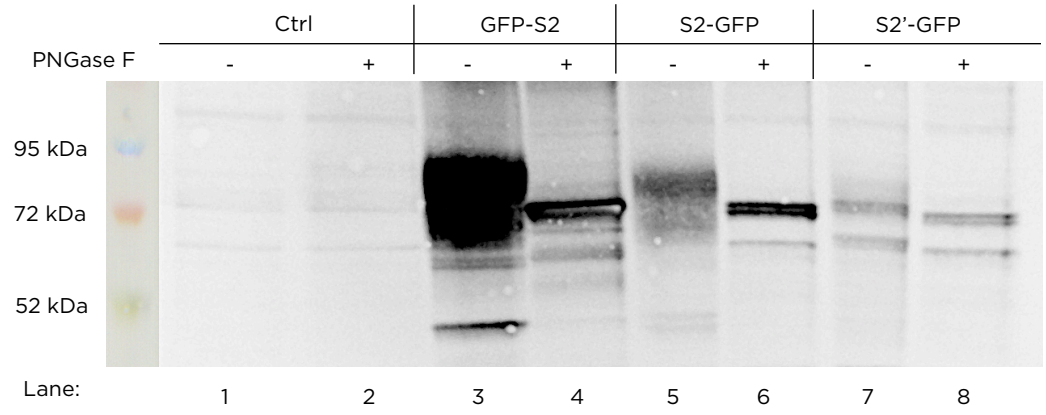

Figure 3.15 - PNGase F treatment of STAMP2 variants in MDCK II cells.
MDCK II cells stably expressing GFP-S2, S2-GFP, or S2'-GFP were grown in cell flasks. The cells were harvested and lysed. 30 µl of the cell lysate was treated with PNGase F prior to application onto a 10 % SDS-PAGE gel and Western blotting with anti-GFP.

Expression levels of the STAMP2 variants differed among the cell lines, but a reduction in the molecular mass could be observed for both GFP-S2 and S2-GFP after PNGase F treatment, signifying the presence of N-glycans on the proteins. Untreated GFP-S2 in lane 3 appeared to have both glycosylated and non-glycosylated versions. No clear reduction of S2'-GFP in molecular mass could be observed after PNGase F treatment, indicating the absence of an N-glycan on this protein.

Glycan analysis of transfected MDCK II cells

It has been shown in preliminary experiments that knocking down STAMP2 by addition of siRNA in LNCaP cells resulted in a reduced amount of incorporated sulfate into secreted macromolecules (Prydz and Saatcioglu groups, unpublished data). Based on these experiments, and the difference in localization of the STAMP2 variants expressed in MDCK II cells, it was of interest to further investigate the synthesis and sulfation of glycans in MDCK II cells expressing the STAMP2 variants.

3.1.7 Sulfation studies of MDCK II cells expressing GFP-S2, S2-GFP, and S2'-GFP

To investigate how the STAMP2 variants influence sulfation of proteoglycans and glycoproteins in MDCK cells, both untransfected MDCK II control cells and stably transfected MDCK II cells were labeled with [^{35}S]-sulfate in microwells for 21 hours, as described in section 2.7.1. The radioactive sulfate incorporated into macromolecules during the incubation period was separated from the free radioactive sulfate by Sephadex G50 fine gel filtration and determined with a liquid scintillation counter. The results are presented in figure 3.16. The media of the control cells contained a higher level of [^{35}S]-sulfated macromolecules compared to the media of the transfected cells, although the relative standard deviation of 22 % is quite large. The level of incorporated radioactive sulfate was reduced in both the media and cell lysates in cells expressing GFP-S2, compared to the other cell lines. S2'-GFP contained a slightly increased amount of radioactive sulfate in the cell lysate compared to S2-GFP. For S2-GFP and S2'-GFP expressing cells, the total production of sulfated macromolecules was not significantly reduced compared to that of untransfectd cells, but a larger fraction of the sulfated macromolecules was recovered from the media.

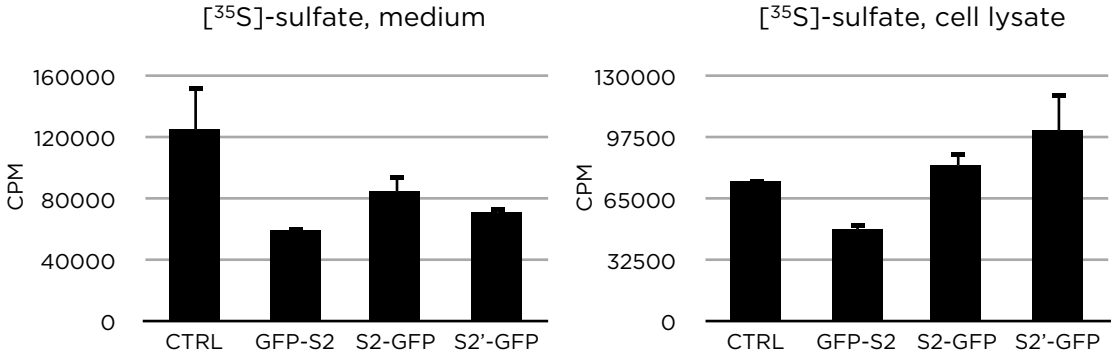

Figure 3.16 - Sulfation studies in MDCK II cells grown in wells.
MDCK II cells stably expressing GFP-S2, S2-GFP or S2'-GFP were grown in microwells and incubated with 0.2 mCi/ml [35S]-sulfate for 21 hours. The media and cell lysates were purified on Sephadex G50 Fine columns before incorporated [35S]-sulfate was detected by liquid scintillation counting. Error bars indicate standard deviation based on three parallels in one experiment. Control; untransfected MDCK II cells.

To further investigate the polarized distribution of the sulfated macromolecules, the cell lines were grown to confluency on filters and labeled with [^{35}S]-sulfate for 22 hours, as described in section 2.7.1. The amount of incorporated radioactive sulfate was determined with a liquid scintillation counter and presented in figure 3.17.

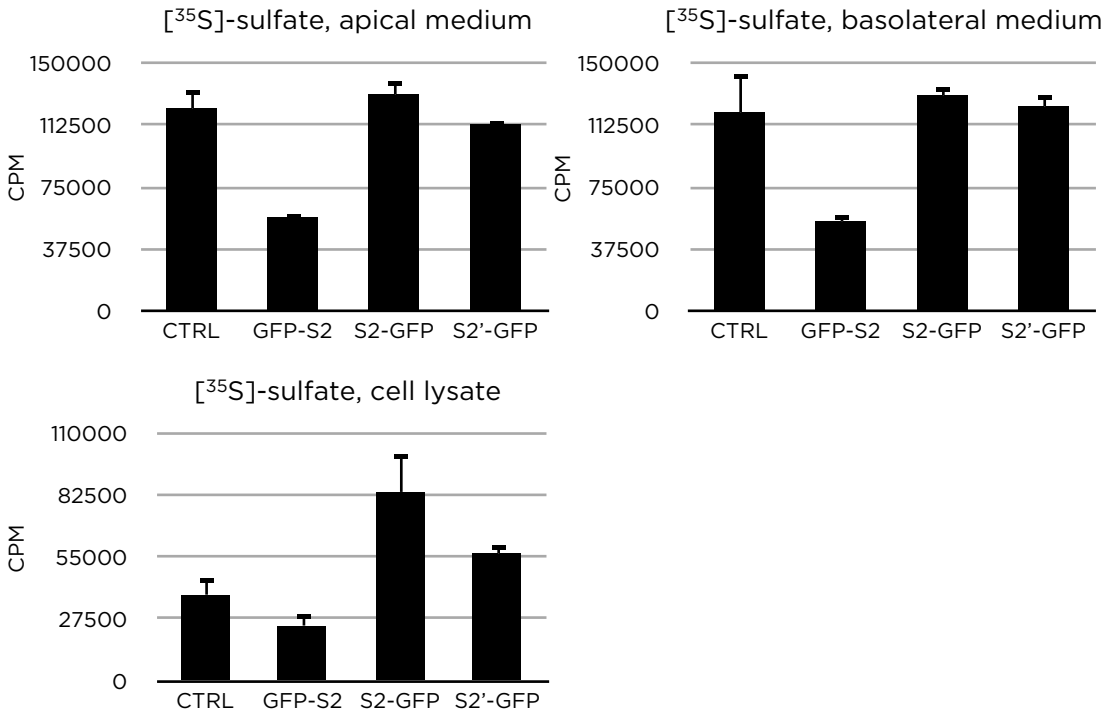

Figure 3.17 - Sulfation studies in MDCK II cells grown on filter.
MDCK II cells stably expressing GFP-S2, S2-GFP and S2'-GFP were grown on filters and incubated with 0.2 mCi/ml [35S]-sulfate for 22 hours. Media and cell lysates were purified on Sephadex G50 Fine columns before incorporated [35S]-sulfate was determined by liquid scintillation counting. Error bars indicate standard deviation based on three parallels in one experiment. Control; untransfected MDCK II cells.

Also for the filter-grown cells, there was a significant reduction in the production of sulfated macromolecules for the GFP-S2 cell line, when compared to the control cells. The secretion of labeled macromolecules from S2-GFP and S2'-GFP cells was comparable to that of untransfected cells, and the amount of labeled macromolecules in the cell fractions of these two cell lines was significantly increased, most dramatically for the S2-GFP cell line.
The results obtained with filter-grown cells were not directly in agreement with those obtained for cells grown in 6-well plates, apart from the evident reduction in the production of sulfated macromolecules observed for GFP-S2 cells. A major difference is that the cells on filters were allowed to form tight monolayers for 3-4 days before radioactive labeling, while the cells grown in wells were labeled the day after plating.

Proteoglycans are frequently observed as a high molecular mass smear in SDS-PAGE gels, while glycoproteins appear as more distinct bands. To study whether radioactive sulfate had been incorporated into glycoproteins or proteoglycans, the radioactively labeled samples from filter-grown cells, were loaded onto a SDS-PAGE gel and the radioactivity was quantified with a phosphorimager screen and analyzed in a Typhoon 9400 phosphorimager (figure 3.18). The amount of sulfate incorporated into PGs (in the upper region of the gel) was dramatically reduced for GFP-S2 cells, while there was no significant reduction for S2-GFP and S2'-GFP.

The increased incorporation observed for S2-GFP and S2'-GFP cell fractions directly after G50 Fine chromatography was not evident for the proteoglycan region of the gel. Such differences could be due to some [^{35}S]-sulfate non-covalently bound to proteins which is removed during SDS-PAGE sample preparation. The amount of sulfated PGs secreted basolaterally was in general substantially higher than apically, while GFP-S2 cells showed a slightly less polarized secretion of PGs than the other cell lines

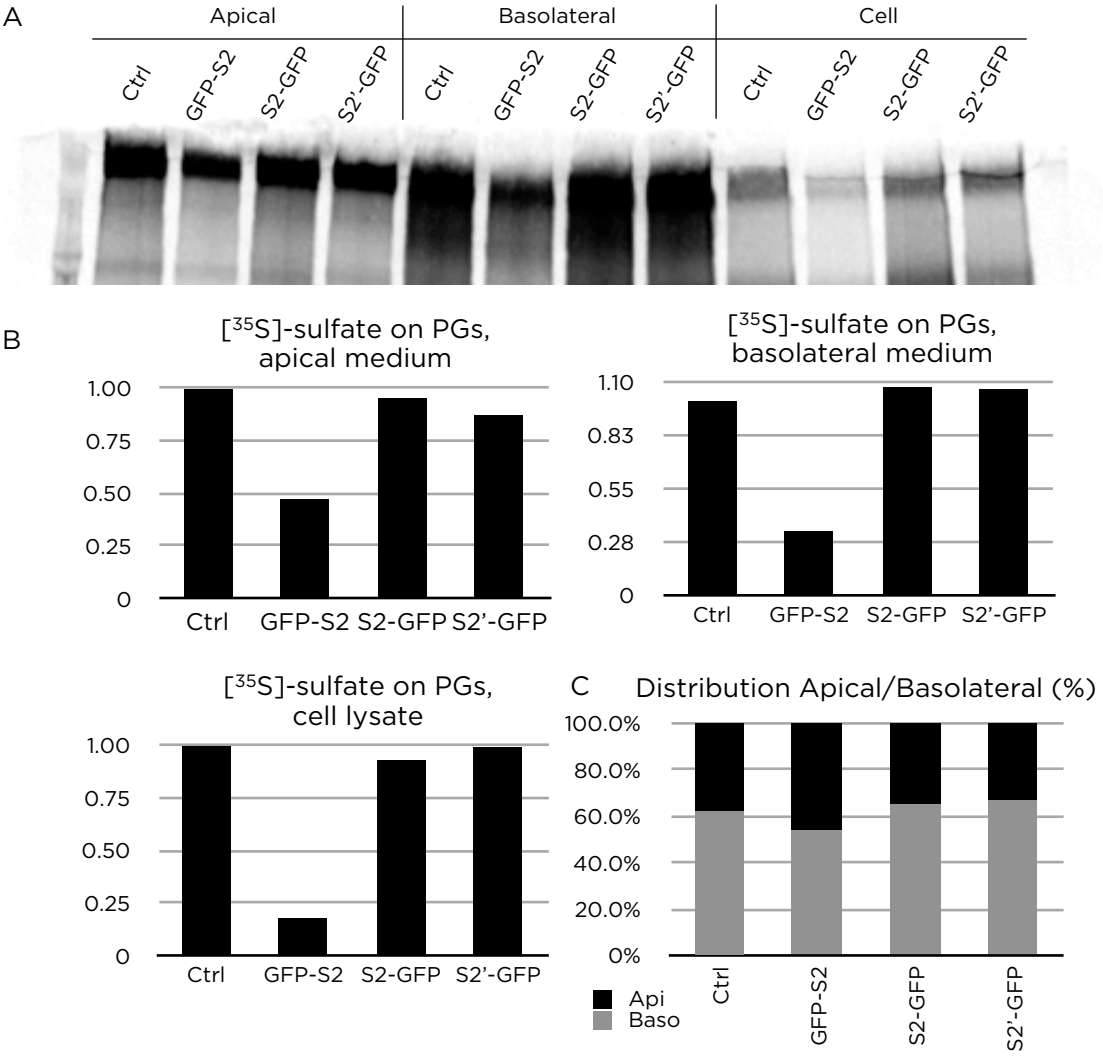

Figure 3.18 - Sulfation of proteoglycans in MDCK II cells stably expressing GFP-S2, S2-GFP or S2'-GFP.

(A) MDCK II cells were grown on filters and labeled with radioactive sulfate for 22 hours. The media and cell lysates were harvested and purified on Sephadex G50 Fine columns before the samples were applied onto 4-12 % SDS-PAGE gels. (B) Quantification of incorporated radioactive sulfate into PGs in (A) using ImageQuant. For (B) the control in each was set as 1. (C) Distribution calculated by dividing the amount of radioactive macromolecules in one medium compartment (apical or basolateral) with the total amount of radioactive macromolecules for both media. Based on results from one experiment

With a 45 % apical, and 55 % basolateral distribution of sulfated PGs, GFP-S2 cells are slightly shifted towards a more apical distribution of sulfated PGs, compared to the control cells, S2-GFP cells, and S2'-GFP cells, which all have a 35 % apical, 65 % basolateral distribution of sulfated PGs. It should be noted that this set of experiments is only performed once.

3.1.8 Glycan studies in MDCK cells expressing GFP-S2, S2-GFP, and S2'-GFP

While radioactive sulfate is mainly incorporated into sulfated GAG chains on PGs, radioactive glucosamine is incorporated into both glycans on glycoproteins and GAG chains. A reduction in the synthesis of GAG chains would be reflected in the amount of radioactive glucosamine in isolated macromolecules, although the effect would be less prominent for glucosamine compared to radioactive sulfate. Cells were grown on filters and labeled with glucosamine for 22 hours, before the samples was purified by G50 Fine chromatography and the amount of radioactivity was determined in a liquid scintillation counter. The results, shown in figure 3.19, show a clear reduction in the amount of incorporated [^{3}H]-glucosamine for GFP-S2 transfected cells. There is also a reduction of incorporated glucosamine in the apical medium of S2'-GFP cells compared to S2-GFP cells and control cells. This reduction of incorporated glucosamine is neither seen for the basolateral medium nor for the cell lysate.

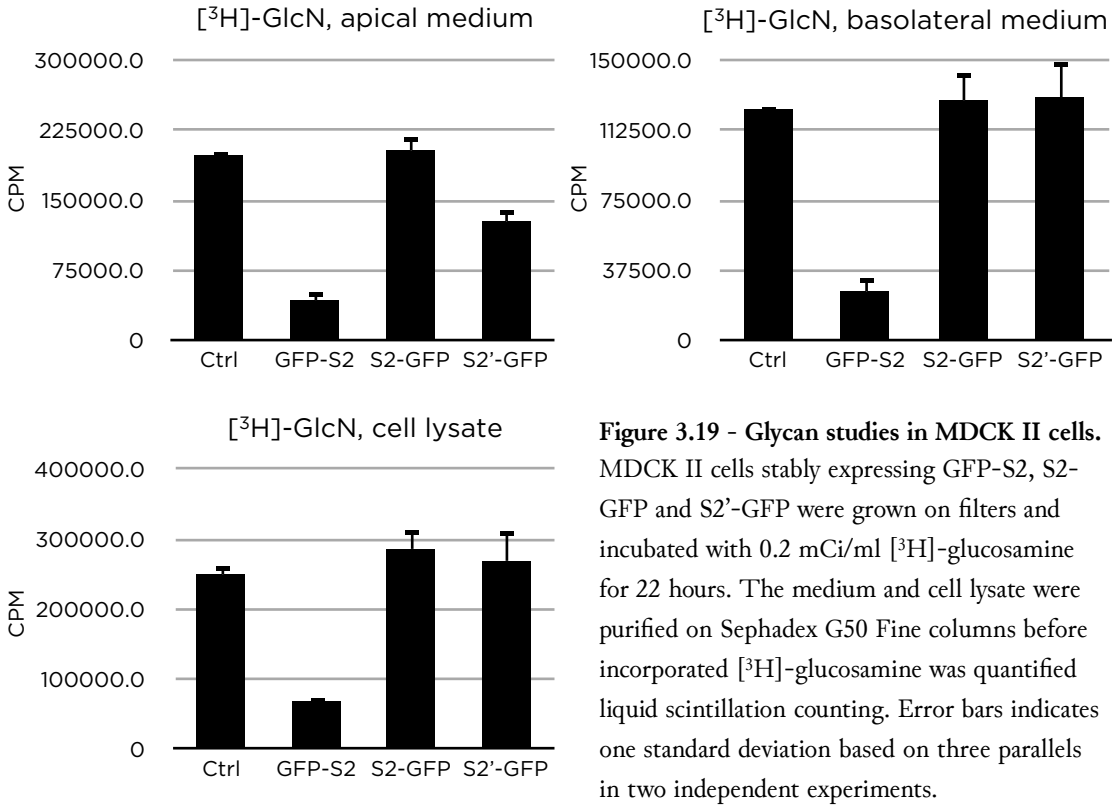

Figure 3.19 - Glycan studies in MDCK II cells. MDCK II cells stably expressing GFP-S2, S2-GFP and S2'-GFP were grown on filters and incubated with 0.2 mCi/ml [^{3}H]-glucosamine for 22 hours. The medium and cell lysate were purified on Sephadex G50 Fine columns before incorporated [^{3}H]-glucosamine was quantified liquid scintillation counting. Error bars indicates one standard deviation based on three parallels in two independent experiments.

3.1.9 Identification of GAG chain type

In order to identify which types of GAG chains that are attached to PGs secreted into the media and present in the cells, aliquots of media and cell lysates from [^{3}H]-glucosamine labeled MDCK II cells stably expressing GFP-S2, S2-GFP and S2'-GFP were treated with cABC or heparinase (sections 2.7.7 and 2.7.8) and loaded on SDS-PAGE gels. cABC degrades CS chains, while heparinase degrades HS chains. Due to their size, PGs are found in the upper part of the gel.

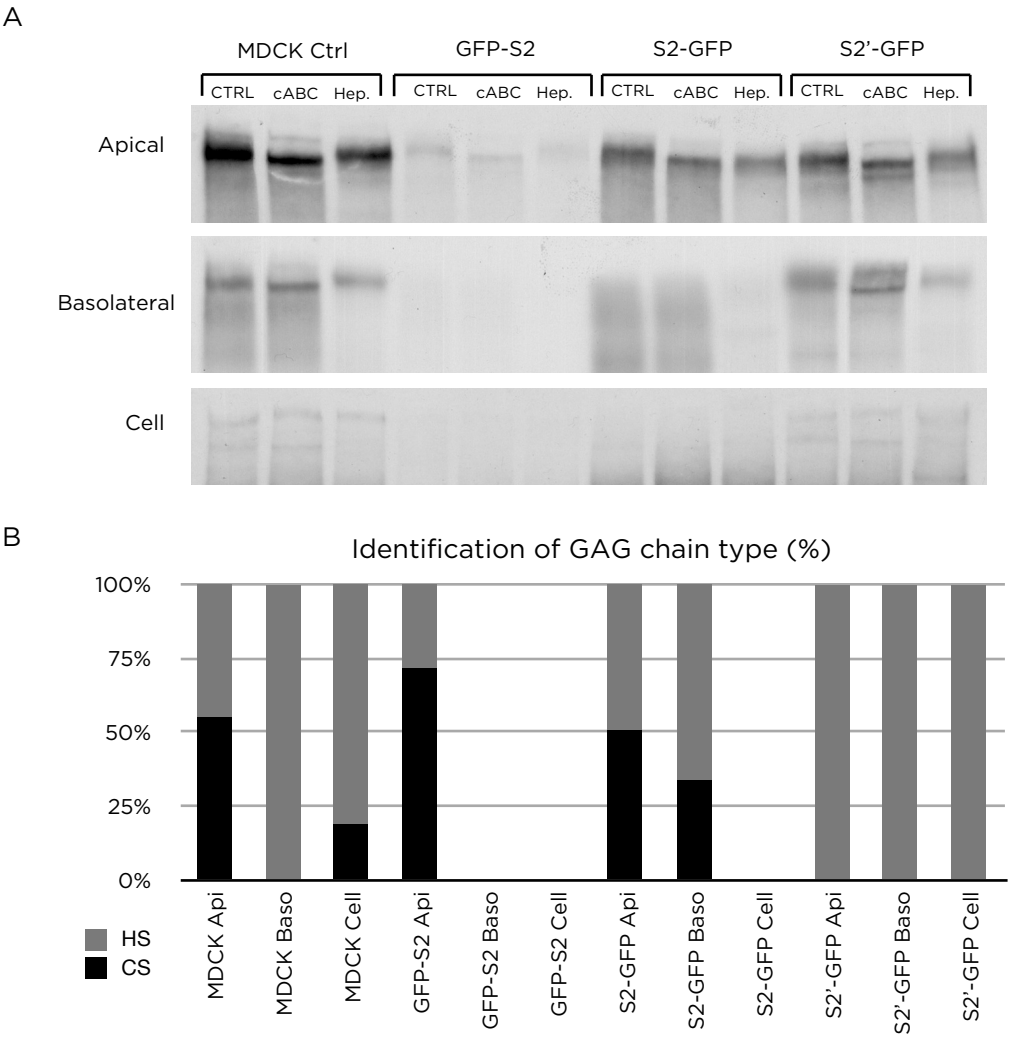

Figure 3.20 - Detection of CS- and HS-GAG chains in MDCK II cells
(A) 100 µl of medium and cell lysate samples from MDCK II cells stably transfected with GFP-S2, S2-GFP and S2'-GFP labeled with radioactive glucosamine were treated with cABC and heparinase before application on 4-12 % SDS-PAGE gels. (B) Quantification of sulfate on PGs in (A) using ImageQuant. Distribution of HS/CS calculated by dividing the reduction in radioactive sulfate by one treatment on the sum of the reduction by both treatments compared to the controls.

As seen in figure 3.20, the majority of the GAG chains secreted basolaterally and in the cell lysate are HS chains. For the untransfected MDCK cells and S2-GFP transfected cells, there is nearly a 50/50 distribution between secreted CS and HS chains in the apical medium. GFP-S2 transfected cells have more secreted CS than HS chains apically, while for S2'-GFP expressing cells have mainly HS chains in the apical medium, since no degradation could be observed with cABC. For the basolateral medium and cell lysate of GFP-S2 and the cell lysate of S2-GFP, the amount of radioactivity in the proteoglycan area of the gel was too low to be detected.

3.1.10 Sulfation studies of GAG chains

To investigate potential differences in the sulfation patterns of CS- and HS-GAG chains, the media and cell lysates from the [^{35}S]-sulfate labeled cells were treated with cABC or heparinase and loaded onto SDS-PAGE gels (figure 3.21). The upper part of the gel containing the PGs is shown.

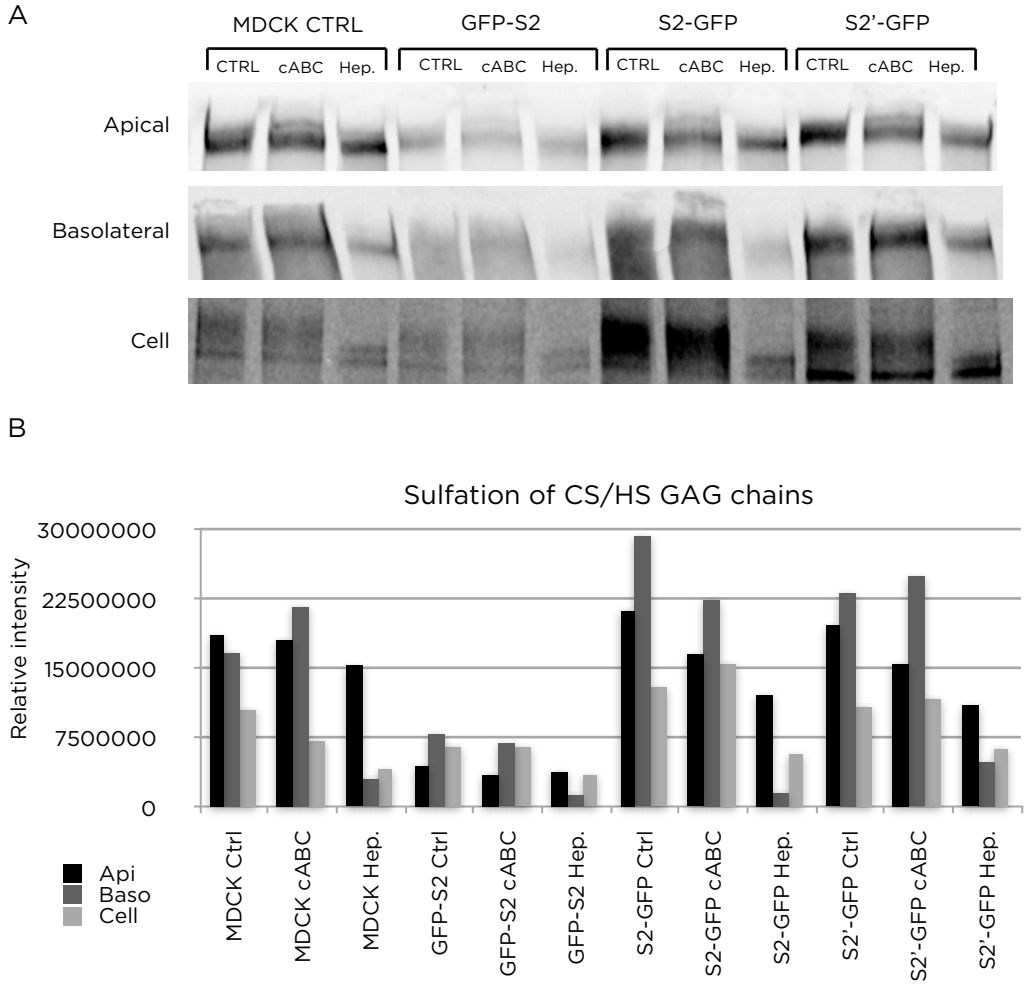

Figure 3.21 - Sufation of GAG chains in MDCK II cells

(A) 100 µl of medium and cell lysate samples labeled with radioactive sulfate was concentrated and treated with cABC and heparinase before being applied on 4-12 % SDS-PAGE gels.

(B) Quantification of sulfate on PGs in (A) using ImageQuant.

There are only minor differences between the cell lines studied with regard to CS/HS ratio, both for the control cells and the STAMP2 expressing cell variants. The majority of the sulfate is incorporated on HS GAG chains. GFP-S2 transfected cells have a much lower level of radioactive sulfate incorporation into GAG chains than the others cell lines, as observed in previous experiments.

3.1.11 *In vitro* GAG synthesis

To investigate whether a faulty GAG synthesis apparatus in the GFP-S2 cells could be the cause of the difference in GAG synthesis and sulfation, isolated Golgi fractions from the different cell lines were incubated with an ATP regenerating system, Mn^{2+}, cytosol, and radioactive sulfate for 2 hours. The samples were then purified on G50 Fine columns, before the amount of incorporated radioactive sulfates into GAG chains was detected by liquid scintillation counting (figure 3.22).

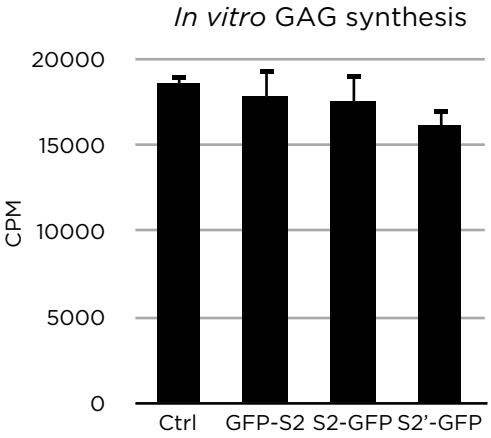

Figure 3.22 - *In vitro* **GAG synthesis**
Golgi fractions isolated by subcellular fractionation were isolated and incubated for 2 hours with 0.2 mCi/mL [^{35}S]-sulfate, an ATP-regenerating system, Mn^{2+}, and with cytosol isolated from pig brain. In vitro synthesized sulfated GAG chains were purified by Sephadex G50 Fine chromatography and incorporated [^{35}S]-sulfate was quantified in a liquid scintillation counter. Error bars represent one standard deviation based on three parallels from from two independent experiments.

None of the Golgi fractions from the different cell lines had a significantly reduced GAG synthesis capacity, eliminating the possibility that an inhibited GAG synthesis apparatus caused the differences seen in GFP-S2 cells, compared to the other cell lines.

3.2 Work with LNCaP cells

Liver cells do not normally express large amounts of STAMP2, but prostate cancer cells do (Korkmaz et al. 2005; Wellen et al. 2007). In preliminary experiments (Prydz and Saatcioglu groups) addition of STAMP2 siRNA to LNCaP cells resulted in a 25-40% reduction in sulfation of macromolecules, while siRNA to STAMP1 did not have a similar effect. To further investigate the possible effect of STAMP2 knock-down on glycan synthesis, we obtained an LNCaP cell line stably expressing shRNA targeting STAMP1, a STAMP2 homolog, and STAMP2 from the Saatcioglu group at IMBV. LNCaP cells stably expressing non-silencing shRNA was used as control. Stable knockdown typically give higher and more stable knockdown and impose less stress on the cells than transient transfections.

3.2.1 Sulfation studies of LNCap cells

Radioactive sulfate is incorporated into GAG chains, and to investigate the level of sulfation in LNCaP cells and LNCaP cells with STAMP1 and STAMP2 knockdown, the cells were grown in microwells and incubated with [^{35}S]-sulfate for 21 hours, before the media and cell lysates were purified by G50 Fine chromatography, and the incorporated sulfate was detected by liquid scintillation counting. As shown in figure 3.23 there was no significant difference in the amounts of incorporated sulfate in the two knockdown cell lines when compared to the control cells. Thus, studies with LNCaP cells with stable knockdown of STAMP2 could not confirm those from studies with transient STAMP2 knock-down.

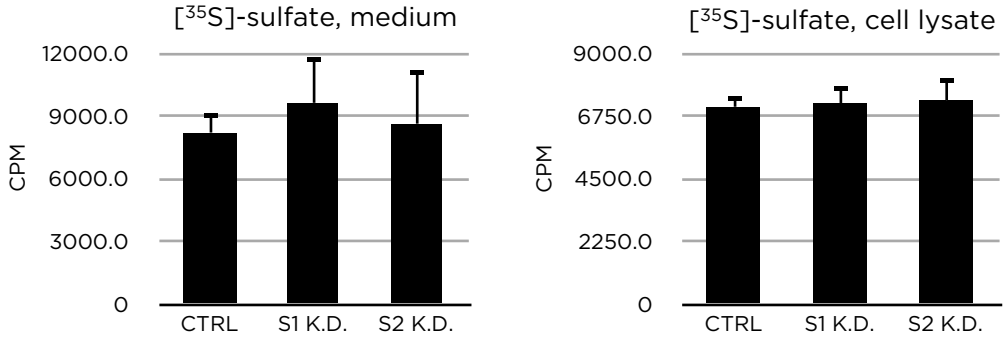

Figure 3.23 - Sulfation studies of LNCaP

LNCaP cells with stable knockdown of endogenous STAMP1 and STAMP2 were grown in microwell plates and incubated with [^{35}S]-sulfate for 21 hours. The media and cell lysates were purified on Sephadex G50 Fine columns before incorporated [^{35}S]-sulfate was determined by liquid scintillation counting. Control set as 1. Error bars represent one standard deviation based on three parallels in two independent experiment. LNCaP cells with non-silencing shRNA were used as control. K.D. = knockdown.

3.2.2 Glycan studies of LNCaP cells

Glucosamine is incorporated into both GAG chains and glycans on glycoproteins. To investigate whether the stable knockdown of STAMP1 or STAMP2 had an effect on synthesis of GAG chains and/or glycoproteins, the cells were grown in microwells and incubated with [^{3}H]-glucosamine for 21 hours, before the media and cell lysates were purified by G50 Fine chromatography, and incorporated sulfate was determined by liquid scintillation counting. As seen in figure 3.24, there was no significant difference in the amount of incorporated glucosamine among the cell lines.

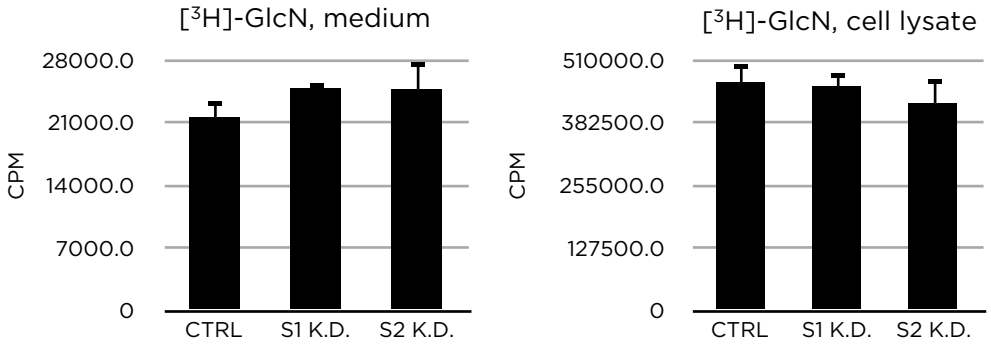

Figure 3.24 - Glycan studies of LNCaP cells
LNCaP cells with stable knockdown of STAMP1 and STAMP2 were grown in microwell plates and incubated with [3H]-glucosamine for 21 hours. The medium and cell lysate were purified on Sephadex G50 Fine columns before incorporated [3H]-glucosamine was determined by liquid scintillation counting. Error bars represent one standard deviation based on three parallels in two experiments. LNCaP cells with non-silencing shRNA were used as control. K.D. = knockdown

4. DISCUSSION

The correct subcellular localization of proteins is often dependent on sorting signals. Signals for proper targeting of transmembrane proteins are often found in their cytoplasmic portion or their localization is determined by the length of the transmembrane domain (Rayner and Pelham 1997; Bonifacino and Traub 2003). An N-terminally GFP-tagged STAMP2 variant had previously been observed in the Golgi apparatus, at the plasma membrane and in VTS in COS-1 cells. Due to the possible blocking of an N-terminal sorting signal by the GFP-tag, two new STAMP2 variants, where the GFP-tag was relocated 20 amino acids in from the N-terminus, were made. One of the two new variants had a three amino acid deletion in the ferric-reductase domain. When these STAMP2 variants were transfected into MDCK II cells they seemed to localize somewhat differently (figure 3.13), as observed by confocal microscopy. GFP-S2, with GFP at the N-terminus, showed a significant co-localization with a lipid-based Golgi marker, but was also detected at the plasma membrane. S2-GFP, with exposed N-terminus, was primarily observed at the plasma membrane and in endosome-like stuctures. S2'-GFP, with exposed N-terminus and a deletion in ORD, was to a large extent observed in the perinuclear area, but with limited co-localizing with the Golgi marker. These results were in agreement with subcellular fractionation experiments with the STAMP2 expressing cells (figure 3.14), in which the STAMP2 variants showed a distribution corresponding to what was seen in the confocal microscope. For GFP-S2 a significant fraction of the recombinant protein localized to the Golgi region, both in the confocal microscope study and in the sucrose gradients. For the variants with the GFP portion deeper into the N-terminal region, there was less observable activity in the Golgi region by both methods.

It has been demonstrated that some transmembrane proteins expressed in epithelial MDCK II cells, lacking basolateral sorting information in their cytoplasmic tail through mutation, may possess N-linked glycans that can act as recessive apical sorting signals. The removal of both apical and basolateral sorting signals can cause an accumulation of the protein in the Golgi apparatus of MDCK cells (Gut et al. 1998). STAMP2 has one predicted N-glycan site (Chen et al. 2009). Both GFP-S2 and S2-GFP were shown to be glycosylated, when expressed in MDCK II cells (figure 3.15). S2'-GFP, however, did not show a significant reduction in molecular mass when treated with PNGase F, an enzyme that cleaves between the innermost GlcNAc and the asparagine residue of N-glycan chains. GFP-S2 localized partly to the Golgi apparatus, despite the presence of an N-glycosylated form. No predominant apical distribution was seen in the confocal microscope (data not shown), indicating that N-glycans did not act as a recessive apical sorting signal for STAMP2. The difference in glycosylation between S2-GFP and S2'-GFP is most likely caused by the deletion of amino acid 310 - 312 in one of the transmembrane domains in the ORD of S2'-GFP. The proximity of the deletion to the

predicted N-glycan site at N323, could suggest a conformational change preventing the transfer of an oligosaccharide onto the asparagine residue.

When filter-grown STAMP2-expressing MDCK II cells were labeled with radioactive sulfate, GFP-S2 had a drastically reduced amount of radioactive sulfate incorporated into macromolecules in both the apical and basolateral media compared to the other cell lines. The control, S2-GFP and S2'-GFP cells had all similar levels of secreted, sulfated macromolecules in the media. In the cell lysates, the amount of radioactive sulfate incorporated into the macromolecular fraction was increased for both S2-GFP and S2'-GFP after Sephadex G50-fine chromatography, but this increase was not observed when the incorporation into PGs was addressed by SDS-PAGE. This could be due to removal of some radioactive sulfate non-covalently bound to proteins (after Sephadex G50-fine chromatography) during sample preparation for SDS-PAGE. For GFP-S2, a further decrease in the incorporation of radioactive sulfate into macromolecules in the cell lysate, compared to the control cells, was observed, when only measuring the amount of radioactivity incorporated in PGs. The distribution of secreted, sulfated PGs was also slightly different for GFP-S2, when compared to the untransfected control, S2-GFP, and S2'-GFP cells, with a slightly higher relative fraction of secreted PGs in the apical medium.

The reduction observed in [^{35}S]-sulfate incorporation into macromolecules for GFP-S2 expressing cells, when compared to control cells, was matched by a similar reduction when the cells were labeled with [^{3}H]-glucosamine. This indicates a reduction in the synthesis of GAG chains, when compared to the other cell lines, and not only a reduction in sulfation. When treating both media and cell lysate samples from each of the cell lines with the CS degrading enzyme cABC, only small reductions in the sulfate levels of PGs could be observed, while after heparinase treatment, there was a more pronounced reduction. This indicates that most of the GAG chains secreted into the media and present in the cell fractions were of HS type. One notable exception was the apical medium of GFP-S2 expressing cells, where there was more CS than HS. Unfortunately, the amount of radioactive glucosamine in the PG area of the gel was below the detection limit for some of the samples. A similar experiment with [^{3}H]-sulfate labeled cell lines showed that the majority of the sulfate was incorporated into HS GAG chains, except for PGs secreted into the apical medium from S2-GFP and S2'-GFP expressing cells, where the macromolecular sulfate is distributed 50/50 between CS and HS chains.

The reduced GAG synthesis in GFP-S2 expressing MDCK cells cannot be explained conclusively. Since STAMP2 is not normally expressed in MDCK cells, a PG recycling circuit would operate in the absence of STAMP2. It has previously been suggested that

the GAG chains of PGs undergo autocleavage followed by resynthesis of the GAG chains, first proposed by Mani et al. (2000). The HS chains on glypican-1 were shown to be cleaved off after endocytosis of the GPI-anchored protein core, before it was recycled to the Golgi apparatus where HS-GAG chains were resynthesized on the protein core. The GAG chain cleavage required copper ions in endosomes (Ding et al. 2002). The reduced GAG synthesis in GFP-S2 expressing MDCK cells could therefore either be caused by reduced cleavage of GAG chains in the endocytic pathway, and thus also a reduced demand for resynthesis, or the localization of GFP-S2 to the Golgi apparatus could have an inhibitory effect on GAG polymerization. In *in vitro* studies, however, the Golgi fractions isolated from all the three novel cell lines and the control MDCK cells had similar capacities for GAG polymerization.

The intracellular localization of endogenously expressed STAMP2 has not been firmly established. The main localization studies were conducted with the GFP-S2 construct expressed with a Golgi localization in this work. Lack of good antibodies has made the localization of the endogenously expressed protein with sufficient resolution difficult. Thus, it can at present not be concluded whether STAMP2 naturally resides in the Golgi apparatus or not, nor which of the expressed constructs that results in the most relevant intracellular expression pattern. Still, it would be of interest to compare the differences in localization of GFP-S2 and S2-GFP more thoroughly. One aspect that also could be interesting to address, is the topology of these two recombinant proteins in the intracellular membranes, to investigate whether different topologies could explain the differences observed for the two cell lines.

Radioactive labeling of LNCaP cells stably transfected with shRNA against STAMP1 or STAMP2, showed no significant difference in the incorporation of [^{3}H]-glucosamine or [^{35}H]-sulfate into macromolecules, compared to control cells stably transfected with non-silencing shRNA. The previously observed effects of STAMP2 knockdown on the incorporation of radioactive sulfate into macromolecules in LNCaP cells were performed with transient knockdown, using siRNA (unpublished results). This could be due to that the stable knockdown cell lines have developed mechanisms to compensate for the loss of STAMP2. The development of compensatory mechanisms in LNCaP cells with stable knockdown for STAMP1 and STAMP2 has previously been reported by Jonson (2009). Culturing of stable STAMP2 knockdown LNCaP cells over time resulted in a selection of cells expressing low levels of shRNA and thus nearly normal levels of STAMP2 were expressed.

In summary, three GFP-tagged STAMP2 variants were transfected into MDCK II cells. The variant with GFP fused to the very N-terminal end localized primarily to the Golgi apparatus, and showed a drastically reduced synthesis and sulfation of GAG chains,

compared to the variants with exposed STAMP2 N-terminus, which were located to the plasma membrane and endosomal compartment.

GAG chains of recycled PGs have been shown to be cleaved off their protein core in endosomes, in a copper-dependent manner, before further transfer of the protein cores to the Golgi apparatus, where new GAG chains are synthesized. A possible role of STAMP2 could be reduction of copper ions for transport into endosomes by the reduction state specific metal transporters DMT1 and CTR1 for cleavage of GAG chains on recycled proteoglycans, but this possibility awaits further investigation.

Further studies

Through the work of this thesis we have obtained indications that STAMP2 can play a role in the cleavage of GAG chains of endocytosed PGs. To investigate this claim, it would be of interest to further study the reductase activity of the deletion mutant of STAMP2, considering the lack of observable difference between the mutant and a non-mutated variant. If the reductase activity is still present, it would be very interesting to repeat the experiments performed in this thesis, using another mutant without reductase activity.

Because of the low levels of S2-GFP and S2'-GFP in the transfected MDCK II cells, repeating the transfection to obtain cell lines with higher expression levels would also be of interest in addressing the role of STAMP2 in PG recycling.

Another interesting prospect would be localization studies without GFP, using immunofluorescence microscopy to detect the true intracellular localization of STAMP2, without concern for abberant localization induced by GFP. Such studies could preferentially be conducted with comparison to endosomal markers, to better study the localization of STAMP2 to different classes of endosomes. Such studies could be conducted both at the immunofluorescence and the electron microscopy level. Though, this would require the development of improved antibodies against STAMP2. The experiments with LNCaP cells could be repeated using cell lines with an inducible knockdown of STAMP1 and STAMP2, within an optimal time frame, to prevent the development of compensatory mechanisms.

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APPENDICES

Appendix 1 - Sequences

Primer sequences:

P201 - STAMP2-F1
ATTGCTAGCGTAATGGAGAAAACTTGTATAGATGCACTTCCTCTTACTATGAATTCTTCAGAAAAGCAAGAGGTGAGCAAGGGCGAGG

P202 - STAMP2-R1
GTTCCAAAAATACATACAGTCTTGTACAGCTCGTCCATGC

P203 - STAMP2-R2
AATCTCGAGCTACTAGTGTTTTGAGTTCCTTTCCC

Red = NheI restriction site
Underlined = Start codon, Met for STAMP2 gene
Blue = EcoRI restriction site
Green = eGFP sequence
Purple = XheI restriction site

STAMP1 amino acid sequence from UniProt (Q8NFT2)

```
        10         20         30         40         50         60
MESISMMGSP KSLSETCLPN GINGIKDARK VTVGVIGSGD FAKSLTIRLI RCGYHVVIGS

        70         80         90        100        110        120
RNPKFASEFF PHVVDVTHHE DALTKTNIIF VAIHREHYTS LWDLRHLLVG KILIDVSNNM

       130        140        150        160        170        180
RINQYPESNA EYLASLFPDS LIVKGFNVVS AWALQLGPKD ASRQVYICSN NIQARQQVIE

       190        200        210        220        230        240
LARQLNFIPI DLGSLSSARE IENLPLRLFT LWRGPVVVAI SLATFFFLYS FVRDVIHPYA

       250        260        270        280        290        300
RNQQSDFYKI PIEIVNKTLP IVAITLLSLV YLAGLLAAAY QLYYGTKYRR FPPWLETWLQ

       310        320        330        340        350        360
CRKQLGLLSF FFAMVHVAYS LCLPMRRSER YLFLNMAYQQ VHANIENSWN EEEVWRIEMY

       370        380        390        400        410        420
ISFGIMSLGL LSLLAVTSIP SVSNALNWRE FSFIQSTLGY VALLISTFHV LIYGWKRAFE

       430        440        450        460        470        480
EEYYRFYTPP NFVLALVLPS IVILGKIILF LPCISRKLKR IKKGWEKSQF LEEGIGGTIP

       490
HVSPERVTVM
```

STAMP2 amino acid sequence from UniProt (Q687X5)

```
        10         20         30         40         50         60
MEKTCIDALP LTMNSSEKQE TVCIFGTGDF GRSLGLKMLQ CGYSVVFGSR NPQKTTLLPS

        70         80         90        100        110        120
GAEVLSYSEA AKKSGIIIIA IHREHYDFLT ELTEVLNGKI LVDISNNLKI NQYPESNAEY

       130        140        150        160        170        180
LAHLVPGAHV VKAFNTISAW ALQSGALDAS RQVFVCGNDS KAKQRVMDIV RNLGLTPMDQ

       190        200        210        220        230        240
GSLMAAKEIE KYPLQLFPMW RFPFYLSAVL CVFLFFYCVI RDVIYPYVYE KKDNTFRMAI

       250        260        270        280        290        300
SIPNRIFPIT ALTLLALVYL PGVIAAILQL YRGTKYRRFP DWLDHWMLCR KQLGLVALGF

       310        320        330        340        350        360
AFLHVLYTLV IPIRYYVRWR LGNLTVTQAI LKKENPFSTS SAWLSDSYVA LGILGFFLFV

       370        380        390        400        410        420
LLGITSLPSV SNAVNWREFR FVQSKLGYLT LILCTAHTLV YGGKRFLSPS NLRWYLPAAY

       430        440        450
VLGLIIPCTV LVIKFVLIMP CVDNTLTRIR QGWERNSKH
```

GFP-S2 sequence

```
ATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCG
GCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGAC
CACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTC
CAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC
TGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAA
GCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGG
TCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGAGAAAACTTGTATAGATGCACTTCCTCTTACTAT
GAATTCTTCAGAAAAGCAAGAGACTGTATGTATTTTTGGAACTGGTGATTTTGGAAGATCACTGGGATTGAAAATGCTCCAGTGTGGTTATTCT
GTTGTTTTTGGAAGTCGAAACCCCCAGAAGACCACCCTACTGCCCAGTGGTGCAGAAGTCTTGAGCTATTCAGAAGCAGCCAAGAAGTCTGGCA
TCATAATCATAGCAATCCACAGAGAGCATTATGATTTTCTCACAGAATTAACTGAGGTTCTCAATGGAAAAATATTGGTAGACATCAGCAACAA
CCTCAAAATCAATCAATATCCAGAATCTAATGCAGAGTACCTTGCTCATTTGGTGCCAGGAGCCCACGTGGTAAAAGCATTTAACACCATCTCA
GCCTGGGCTCTCCAGTCAGGAGCACTGGATGCAAGTCGGCAGGTGTTTGTGTGTGGAAATGACAGCAAAGCCAAGCAAAGAGTGATGGATATTG
TTCGTAATCTTGGACTTACTCCAATGGATCAAGGATCACTCATGGCAGCCAAAGAAATTGAAAAGTACCCCCTGCAGCTATTTCCAATGTGGAG
GTTCCCCTTCTATTTGTCTGCTGTGCTGTGTGTCTTCTTGTTTTTCTATTGTGTTATAAGAGACGTAATCTACCCTTATGTTTATGAAAAGAAA
GATAATACATTTCGTATGGCTATTTCCATTCCAAATCGTATCTTTCCAATAACAGCACTTACACTGCTTGCTTTGGTTTACCTCCCTGGTGTTA
TTGCTGCCATTCTACAACTGTACCGAGGCACAAAATACCGTCGATTCCCAGACTGGCTTGACCACTGGATGCTTTGCCGAAAGCAGCTTGGCTT
GGTAGCTCTGGGATTTGCCTTCCTTCATGTCCTCTACACACTTGTGATTCCTATTCGATATTATGTACGATGGAGATTGGGAAACTTAACCGTT
ACCCAGGCAATACTCAAGAAGGAGAATCCATTTAGCACCTCCTCAGCCTGGCTCAGTGATTCATATGTGGCTTTGGGAATACTTGGGTTTTTTC
TGTTTGTACTCTTGGGAATCACTTCTTTGCCATCTGTTAGCAATGCAGTCAACTGGAGAGAGTTCCGATTTGTCCAGTCCAAACTGGGTTATTT
GACCCTGATCTTGTGTACAGCCCACACCCTGGTGTACGGTGGGAAGAGATTCCTCAGCCCTTCAAATCTCAGATGGTATCTTCCTGCAGCCTAC
GTGTTAGGGCTTATCATTCCTTGCACTGTGCTGGTGATCAAGTTTGTCCTAATCATGCCATGTGTAGACAACACCCTTACAAGGATCCGCCAGG
GCTGGGAAAGGAACTCAAAACACTAGTAGCTCGAG
```

Green = eGFP

Blue = EcoRI restriction site

Orange = nucleotides deleted in S2'-GFP

Purple = XheI restriction site

S2-GFP sequence

```
ATTGCTAGCGTAATGGAGAAAACTTGTATAGATGCACTTCCTCTTACTATGAATTCTTCAGAAAAGCAAGAGGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA
CGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGC
TTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGG
ACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGA
CGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAC
TTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGC
CCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGC
CGGGATCACTCTCGGCATGGACGAGCTGTACAAGACTGTATGTATTTTTGGAACTGGTGATTTTGGAAGATCACTGGGATTGAAAATGCTCCAG
TGTGGTTATTCTGTTGTTTTTGGAAGTCGAAACCCCCAGAAGACCACCCTACTGCCCAGTGGTGCAGAAGTCTTGAGCTATTCAGAAGCAGCCA
```

AGAAGTCTGGCATCATAATCATAGCAATCCACAGAGAGCATTATGATTTTCTCACAGAATTAACTGAGGTTCTCAATGGAAAAATATTGGTAGA
CATCAGCAACAACCTCAAAATCAATCAATATCCAGAATCTAATGCAGAGTACCTTGCTCATTTGGTGCCAGGAGCCCACGTGGTAAAAGCATTT
AACACCATCTCAGCCTGGGCTCTCCAGTCAGGAGCACTGGATGCAAGTCGGCAGGTGTTTGTGTGTGGAAATGACAGCAAAGCCAAGCAAAGAG
TGATGGATATTGTTCGTAATCTTGGACTTACTCCAATGGATCAAGGATCACTCATGGCAGCCAAAGAAATTGAAAAGTACCCCCTGCAGCTATT
TCCAATGTGGAGGTTCCCCTTCTATTTGTCTGCTGTGCTGTGTGTCTTCTTGTTTTTCTATTGTGTTATAAGAGACGTAATCTACCCTTATGTT
TATGAAAAGAAAGATAATACATTTCGTATGGCTATTTCCATTCCAAATCGTATCTTTCCAATAACAGCACTTACACTGCTTGCTTTGGTTTACC
TCCCTGGTGTTATTGCTGCCATTCTACAACTGTACCGAGGCACAAAATACCGTCGATTCCCAGACTGGCTTGACCACTGGATGCTTTGCCGAAA
GCAGCTTGGCTTGGTAGCTCTGGGATTTGCCTTCCTTCATGTCCTCTACACACTTGTGATTCCTATTCGATATTATGTACGATGGAGATTGGGA
AACTTAACCGTTACCCAGGCAATACTCAAGAAGGAGAATCCATTTAGCACCTCCTCAGCCTGGCTCAGTGATTCATATGTGGCTTTGGGAATAC
TTGGGTTTTTTCTGTTTGTACTCTTGGGAATCACTTCTTTGCCATCTGTTAGCAATGCAGTCAACTGGAGAGAGTTCCGATTTGTCCAGTCCAA
ACTGGGTTATTTGACCCTGATCTTGTGTACAGCCCACACCCTGGTGTACGGTGGGAAGAGATTCCTCAGCCCTTCAAATCTCAGATGGTATCTT
CCTGCAGCCTACGTGTTAGGGCTTATCATTCCTTGCACTGTGCTGGTGATCAAGTTTGTCCTAATCATGCCATGTGTAGACAACACCCTTACAA
GGATCCGCCAGGGCTGGGAAAGGAACTCAAAACACTAGTAGCTCGAG

Red = NheI restriction site

Green = eGFP

Blue = EcoRI restriction site

Orange = nucleotides deleted in S2'-GFP

Purple = XheI restriction site

S2'-GFP sequence

ATTGCTAGCGTAATGGAGAAAACTTGTATAGATGCACTTCCTCTTACTATGAATTCTTCAGAAAAGCAAGAGGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTA
CGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGC
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ACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGA
CGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAAC
TTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGC
CCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGC
CGGGATCACTCTCGGCATGGACGAGCTGTACAAGACTGTATGTATTTTTGGAACTGGTGATTTTGGAAGATCACTGGGATTGAAAATGCTCCAG
TGTGGTTATTCTGTTGTTTTTGGAAGTCGAAACCCCCAGAAGACCACCCTACTGCCCAGTGGTGCAGAAGTCTTGAGCTATTCAGAAGCAGCCA
AGAAGTCTGGCATCATAATCATAGCAATCCACAGAGAGCATTATGATTTTCTCACAGAATTAACTGAGGTTCTCAATGGAAAAATATTGGTAGA
CATCAGCAACAACCTCAAAATCAATCAATATCCAGAATCTAATGCAGAGTACCTTGCTCATTTGGTGCCAGGAGCCCACGTGGTAAAAGCATTT
AACACCATCTCAGCCTGGGCTCTCCAGTCAGGAGCACTGGATGCAAGTCGGCAGGTGTTTGTGTGTGGAAATGACAGCAAAGCCAAGCAAAGAG
TGATGGATATTGTTCGTAATCTTGGACTTACTCCAATGGATCAAGGATCACTCATGGCAGCCAAAGAAATTGAAAAGTACCCCCTGCAGCTATT
TCCAATGTGGAGGTTCCCCTTCTATTTGTCTGCTGTGCTGTGTGTCTTCTTGTTTTTCTATTGTGTTATAAGAGACGTAATCTACCCTTATGTT
TATGAAAAGAAAGATAATACATTTCGTATGGCTATTTCCATTCCAAATCGTATCTTTCCAATAACAGCACTTACACTGCTTGCTTTGGTTTACC
TCCCTGGTGTTATTGCTGCCATTCTACAACTGTACCGAGGCACAAAATACCGTCGATTCCCAGACTGGCTTGACCACTGGATGCTTTGCCGAAA
GCAGCTTGGCTTGGTAGCTCTGGGATTTGCCTTCCTTCATGTCCTCTACACACTTATTCGATATTATGTACGATGGAGATTGGGAAACTTAACC
GTTACCCAGGCAATACTCAAGAAGGAGAATCCATTTAGCACCTCCTCAGCCTGGCTCAGTGATTCATATGTGGCTTTGGGAATACTTGGGTTTT
TTCTGTTTGTACTCTTGGGAATCACTTCTTTGCCATCTGTTAGCAATGCAGTCAACTGGAGAGAGTTCCGATTTGTCCAGTCCAAACTGGGTTA
TTTGACCCTGATCTTGTGTACAGCCCACACCCTGGTGTACGGTGGGAAGAGATTCCTCAGCCCTTCAAATCTCAGATGGTATCTTCCTGCAGCC
TACGTGTTAGGGCTTATCATTCCTTGCACTGTGCTGGTGATCAAGTTTGTCCTAATCATGCCATGTGTAGACAACACCCTTACAAGGATCCGCC
AGGGCTGGGAAAGGAACTCAAAACACTAGTAGCTCGAG

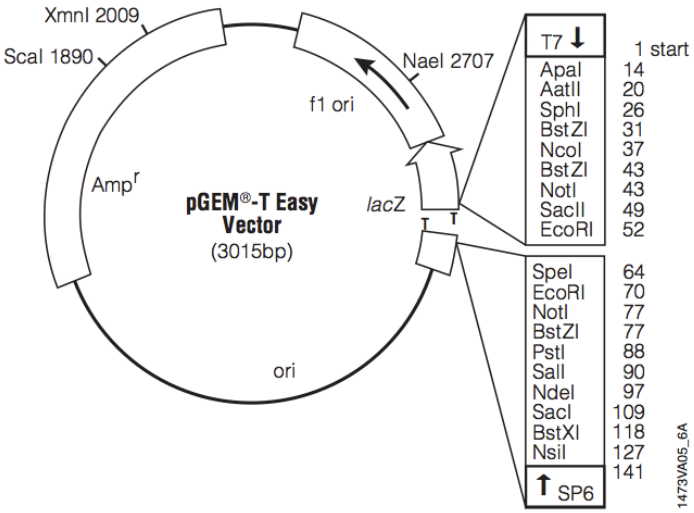
Red = NheI restriction site

Green = eGFP

Blue = EcoRI restriction site

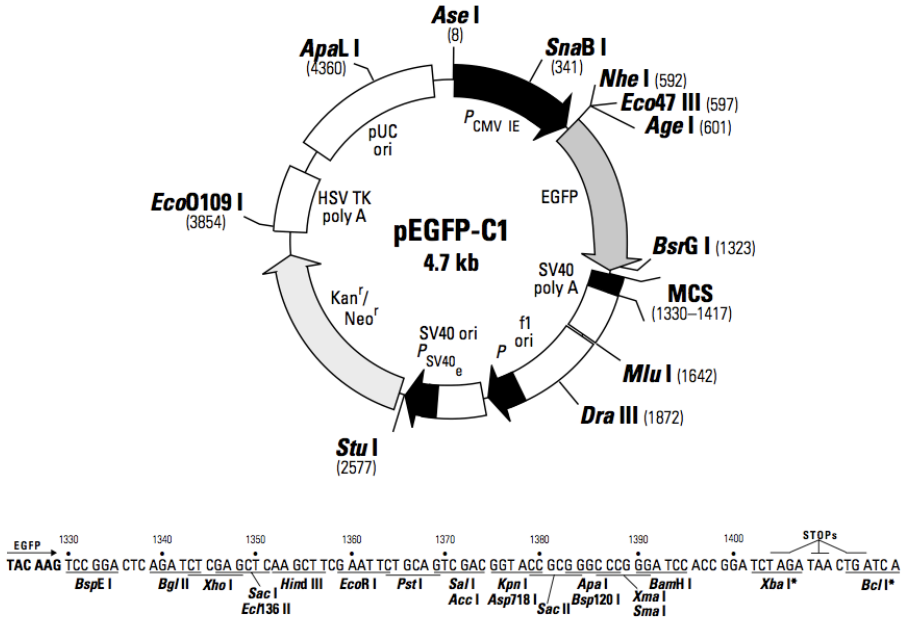
Purple = XheI restriction site

pGEM-T Easy plasmid map



pGEM-T Easy vector (Promega) contains a gene for ampicillin resistance for selection in *E. coli*.

pEGFP-C1 plasmid map



pEGFP-C1 expression vector (Clontech) is a eukaryotic expression vector with a CMS promotor. The vector contains a gene for kanamycin resistance for selection in *E. coli* and G418 selection in stably transfected eukaryotic cells.

Amino acid abbreviations

Amino Acid	3-Letter	1 Letter
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix 2 - Materials

Table 1 - Reagents

Reagent	Manufacturer	Cat. No.	Used for
DMEM (Dulbecco's Modified Eagle's Medium)	Lonza	BE12-614F	Cell culture
DMSO (Dimethyl sulfoxide)	Duchefa Biochemie	D1370.0250	Cell culture
FuGene 6 Transfection Reagent	Roche	11 815 091 001	Cell culture
G418	Sigma	A1720	Cell culture
G418	Duchefa Biochemie	G0175.0005	Cell culture
L-Glutamin	Lonza	17-605E	Cell culture
Pen-Strep	Lonza	17-602E	Cell culture
RPMI-1640 medium	Invitrogen	041-90985M	Cell culture
Sephadex G50 Fine	GE Healthcare	17-0042-02	Cell culture
Trypsin-EDTA	Gibco	25300-104	Cell culture
Ultima Gold XR	Perkin Elmer	6013119	Cell culture
1 kb DNA Ladder, GeneRuler	Fermentas	SM0311	Cloning
6X Loading Dye Solution	Fermentas	R0611	Cloning
Acetic Acid	Merck	K38877863 825	Cloning
Ampicillin sodium salt	Sigma	A0166-25G	Cloning
Bacto-Agar	DB	214010	Cloning
Chloroform	Prolabo	22 711.290	Cloning
Difco LB Broth	BD	244620	Cloning
dNTP	Fermentas	R0149, R0159, R0169, R0179	Cloning
EDTA	Sigma	E5134-500G	Cloning
Ethidium bromide	Sigma	E1510	Cloning
Glycerol	VWR	24388.295	Cloning
Kanamycin sulfate	Sigma	K1377-5G	Cloning
pGEM T-Easy Vector System	Promega	A1360	Cloning
Tris	VWR	33621.26	Cloning
UltraPure Agarose	Invitrogen	15510-027	Cloning
BODIPY TR ceramide	Invitrogen	D-7540	Confocal microscopy
Hoechst 33258, pentahydrate	Invitrogen	H3569	Confocal microscopy
ATP	Sigma	A2383	*In vitro* GAG synthesis
Creatine phosphokinase	Sigma	C6638	*In vitro* GAG synthesis
Phosphocreatine	Sigma	P7936	*In vitro* GAG synthesis
Amplify	GE Healthcare	NAMP100V	SDS-PAGE
Benchmark Fluorescent Protein Standard	Invitrogen	LC5928	SDS-PAGE
XT MOPS Running Buffer	BioRad	161-0788	SDS-PAGE
XT Reducing Agent	BioRad	161-0792	SDS-PAGE
XT Sample Buffer (4X)	BioRad	161-0791	SDS-PAGE
Sucrose	Merck	K33729587 505	Subcellular fractionation
10X Tris/CAPS buffer	BioRad	161-0778	WB
Blotting Grade Blocker Non-Fat Dry Milk	BioRad	170-6404	WB
ECF Substrate for Western Blot	GE Healthcare	RPN5785	WB
Polyoxyethylene-sorbitan monolaureate (Tween 20)	Sigma	P-7949	WB

Table 2 - Restriction enzymes

Restriction enzymes and buffers	Manufacturer	Cat. No.
BSA (100X)	NEB	B9001S
Buffer 2 (10X)	NEB	B7002S
EcoRI	NEB	R0101S
EcoRI Buffer	NEB	B0101S
NheI	NEB	R0131S
XhoI	NEB	R0146S

Table 3 - Other enzymes

Other Enzymes	Manufacturer	Cat. No.
10X T4 DNA Ligase Buffer	Fermentas	B69
Advantage® 2 Polymerase Mix (Enzyme + Buffer)	Clontech	639201
cABC	Seikagaku Corp.	100332
PNGase F	NEB	P0705S
T4 DNA Ligase	Fermentas	EL0014

Table 4 - Kits

Kits	Manufacturer	Cat. No.
QIAquick Gel Extraction Kit	QIAGEN	28704
QIAquick Spin Miniprep Kit	QIAGEN	27104
HiSpeed Plasmid Midi Kit	QIAGEN	12643
NucleoSpin Plasmid	Machery Nagel	740 588.250
NucleoSpin Extract II	Machery Nagel	740 609.50

Table 5 - Antibodies

Antibodies	Manufacturer	Cat. No.
Rb pAb to GFP	Abcam	ab6556-25
Anti-rabbit IgG alikaline phosphatase linked whole antibody (from goat)	GE Healthcare	NIF1317

Table 6 - Radioactive isotopes

Radioactive isotopes	Manufacturer	Cat. No.
[^{35}S]Sulphuric acid	Hartmann Analytic	126979
[^{3}H]Glucosamine HCl	American Radiolabeled Chemicals Inc.	0110A

Table 7 - Disposable equipment

Disposable equipment	Manufacturer	Cat. No.
10 cm^2 petri dish	Sarstedt	
10 ml syringe	BD	302188

Disposable equipment	Manufacturer	Cat. No.
15 ml tubes	Sarstedt	
50 ml tubes	Sarstedt	
6 well culture cluster	Costar	3516
Amersham Hyperfilm MP	GE Healthcare	28906843
BD Microlance 3 22 ½ G needle	BD	301000
Cell flasks		
Criterion XT Precast Gel 10 % Bis-Tris	BioRad	345-0111
Criterion XT Precast Gel 4-12 % Bis-Tris	BioRad	345-0123
Cryogenic vial	Corning	430488
Extra thick Blotting paper	BioRad	1703967
Glass bottom microwell dish	MatTek Corp.	P35G-1.5-14-C
Hybond™-P PVDF membrane	GE Healthcare	RPN303F
Microfuge tube	Sarstedt	
Sterile 5, 10 and 25 ml pipettes	Sarstedt	
Transwell Permeable Support 0.4 µl polycarbonate membrane	Costar	3412
Ultra-Clear Centrifuge Tubes	Beckman	344085

Table 8 - Equipment

Equipment	Manufacturer
Agarose gel electrophoresis chamber	VWR
Autoclave SS-325	TOMY
Cell incubator	
Centrifuge 5415R	Eppendorf
Confocal microscope IX81	Olympus
Dri-Block DB-2D	Techne
Gel electrophoresis power supply	Pharmacia
GS-15R Centrifuge	Beckman
Liquid scintillation analyzer 1900 TR	Packard
miniSpin	Eppendorf
NanoDrop-1000	Thermo Scientific
Optima LE-80K Ultracentrifuge	Beckman Coulter
Peltier Thermal Cycler-200	BioRad
pH meter 420	Thermo
Trans-Blot Semi-Dry Electrophoretic Transfer Cell	BioRad
Typhoon 9400 Variable Mode Imager	GE Healthcare
UV transilluminator	UVI Tec

Appendix 3 - Solutions

MilliQ (MQ) water
Ultra-pure distilled water purified by the Millipore MQ system and autoclaved before use.

LB medium
Add 20 g LB broth to 1000 ml MQ water. Autoclave solution and let it cool down to 50 °C before adding 500 µl of ampicillin (50 µg/ml) or kanamycin (50 µg/ml)

LB plates
Make LB medium and add 1.5 % bactoagar before autoclaving. Let the solution cool down to 50 °C before adding antibiotics. Transfer solution to petri dishes and stack the dishes upside down to prevent condensation. Store in sealed plastic bags at 4 °C.

SOB medium
Mix 10 g Trypton, 2.5 g yeast extract, 0.25 g NaCl and 415 µl KCl (3 M) in 500 ml MQ water and adjust pH to 7.0. Autoclave solution. Add 2.5 ml sterile $MgCl_2$ (2 M) before use.

EDTA (0.5 M)
Add 93.05 g EDTA disodium salt to 400 ml MQ water. Adjust pH to 8.0 using NaOH. Add MQ water to a final volume of 500 ml.

50X TAE
Mix 242 g Tris base, 57.1 ml acetic acid and 100 ml EDTA (0.5 M) in 750 ml distilled water and adjust pH to 8.0. Adjust volume to 1 l using distilled water.

1 M PIPES
Dissolve 342.4 g PIPES in 1 l MQ water and autoclave before use.

Transformation buffer (TB)
Mix 10 mM PIPES, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$. Adjust pH to 6.7 using 5 M KOH.

Lysis buffer
Mix 1 % (5 g) Nonidet P-40, 50 mM (25 ml of 1M) Tris (pH 7.5), 2 mM (0.3722 g) EDTA, 150 mM (18.75 ml of 4M) NaCl and 35 µg/ml (17.5 mg) PMFS. Adjust volume to 500 ml using MQ water.

SDS-PAGE

XT-MOPS Running buffer
Add 50 ml XT-MOPS (20X) to 950 ml dH_2O.

Western blotting

Cathode buffer
Mix 100 ml Tris/CAPS buffer (10X), 10 ml 10% SDS, and 890 ml dH_2O.

Anode buffer
Mix 100 ml Tris/CAPS buffer (10X), 150 ml methanol, and 750 ml dH_2O.

10X TBS
Mix 80 g NaCl, 200 ml 1 M Tris-HCl (pH 7.4) and 1M000 ml dH_2O.

1X TTBS
Mix 100 ml 10X TBS, 1 ml Tween-20 and 900 ml dH_2O.

Subcellular fractionation

Homogenization buffer
Add 25 mM (42.78 g) sucrose and 3 mM (0.102 g) imidazole to 500 ml dH_2O and adjust pH to 7.4.

1.3 M Sucrose solution
Add 1.3 M (44.5 g) sucrose, 15 mM (0.25 g) CsCl, and 1 mM (0.024 g) HEPES to 100 ml dH_2O.

1.15 M Sucrose solution
Add 1.15 M (39.36 g) sucrose, 15 mM (0.25 g) CsCl, and 1 mM (0.024 g) HEPES to 100 ml dH_2O.

2 M Sucrose solution
Add 2 M (68.46 g) sucrose, 10 mM (0.168 g) CsCl, and 1 mM (0.024 g) HEPES to 100 ml dH_2O.

0.9 M Sucrose solution
Add 0.9 M (30.81 g) sucrose and 1 mM (0.024 g) HEPES to 100 ml dH_2O.