TIP47 is recruited to lipid droplets and important for the organelle biogenesis and function

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To my grandfather.

Content

Abbreviations

Abbreviations

ACS	acyl-CoA synthetase
ADRP	Adipose differentiation related protein
Amp	Ampicillin
AP	Adaptor Protein
APS	Ammoniumperoxodisulfate
ARF	Adenosine 5'-diphosphate Ribosylation Factor
BSA	Bovine Serum Albumin
cAMP	Adenosine 3',5'- cyclic monophosphate
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
ddH_2O	double distilled water
ddNTPs	di-desoxynucleotides
DGAT	Acyl-CoA-diacylglycerol acyltransferase CoA
DMEM	Dulbecco's Modified Eagles Medium
DMS	Dimethylsulfoxide
DNA	Deoxyribonucleicacid
dNTPs	Deoxynucleosidetriphosphate (dATP, dGTP, dCTP, dTTP)
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediamintetraacetate-Disodium salt
ER	Endoplasmic Reticulum
EtOH	Ethanol
FACL	Fatty acid (long chain) CoA ligase
FABP	Fatty acid binding protein
FATP	Fatty acid transport protein
FCS	Fetal Calf Serum
GFP	Green fluorescent protein
GGA	Golgi-localized Gamma ear domain-containing ARF binding proteins

GST	Glutathione-S-Transferase
GTP	Guanosine 5'-triphosphate
IIF	Indirect immunofluorescence
h	Hour
HeLa	Henrietta Lacks
HEPES	N-2-Hydroxyethylpiperazin-N'-2-ethanesulfonic acid
HRP	Horseradish-Peroxidase
HSL	Hormone sensitive lipase
HSP	Heat shock protein
kb	Kilobase
kD	Kilodalton
l	Liter
LB	Luria Bertani
LD	Lipid droplet
M	Molar
mA	Milliampere
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
MPR	Mannose 6-phosphate receptor
MS	Mass spectrometry
NLSD	Neutral lipid storage disease
NSF	N-ethylmaleimide Sensitive Factor
OA	Oleic acid
OD	Optical density
PAGE	Poly-acrylamide gel electrophoresis
PAT family	Perilipin, ADRP and TIP47 family
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PFA	Paraformaldehyde

pH	Negative logarithm of H^+ concentration
PKA	Protein Kinase A
PM	Plasma Membrane
PMSF	Phenylmethylsulfonylfluoride
RNA	Ribonucleicacid
RNAi	RNA interference
s	Seconds
SDS	Sodium Dodecyl Sulfate
siRNA	Small interfering RNA
SNARE	Soluble N-ethylmaleimide Attachment protein Receptor
TAE	Tris-Acetate-EDTA-Buffer
TAG	Triacylglycerol
TCA	Trichloroacetic acid
TE	Tris-EDTA-Buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TIP47	Tail interacting protein of 47kD
TGN	*trans*-Golgi network
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit
UV	Ultraviolet
V	Volt
VLDL	Very low density lipoprotein
v/v	(volume/volume)
Vti1a	Vps10p interacting
w/v	(weight/volume)
wt	Wild type
YFP	Yellow fluorescent protein
α-SNAP	α-soluble NSF attachment protein
µ	Micro

1 Introduction

1.1 Lipid droplets biology

Lipid droplet (LD) biogenesis and turnover take place in cells of most, if not all, mammalian tissues and are an integral part of lipid metabolism. LDs are organelles of 0.05-200μm diameter (Murphy, 2001) which accumulate lipids for both, long- and short-term storage. LDs are found in different types of organisms, ranging from yeast to humans, they share many features and consist of a core of neutral lipids, mainly triacylglycerol and cholesteryl esters as well as some other components, depending on species and cell type (reviewed in (Zweytick et al., 2000)). In contrast to other organelles, LDs are surrounded by a phospholipid monolayer (Leber et al., 1994; Tauchi-Sato et al., 2002). LDs of mammalian cells are characterized by a set of associated proteins, among which the perilipins and the adipose differentiation related protein (ADRP) or adipophilin are the best characterized.

1.1.1 LDs distribution and function

Adipose tissue is the biggest body store of triacylglycerols, which is an important energy source. There are two types of adipose tissue, white and brown, which have been generally regarded as having different physiological functions (triacylglycerol storage and termogenesis, respectively) (Cousin et al., 1993). However, adipose tissue is not the only place where neutral lipids are accumulated. Among other neutral lipids stores of the body are steroidogenic tissues, where lipids accumulate in LDs serve as precursors for synthesis of steroid hormones. LDs can also be found in the heart (Christiansen and Jensen, 1972) and skeletal muscles (Di Mauro et al., 1980; Hulbert et al., 1979), in hepatocytes (Chao et al., 1986), in enterocytes, in epithelial cells of the mammary gland (Ghosal et al., 1994), in several types of blood cells, such as macrophages (Dvorak et al., 1983), eosinophils and neutrophils where LDs play a key role in the arachidonic acid metabolism important for the inflammatory response (Bozza et al., 1997; Dvorak et al., 1994; Weller et al., 1999).

LDs are classically recognised as storage organelles, but their function and significance for the homeostasis of a single cell and the whole body are more complex than originally thought. In addition to the deposition of energy and precursors for the biosynthesis of membrane phospholipids, hormones and

secondary messengers, they are also important for the maintenance of the level of free unesterified fatty acids and cholesterol to avoid their toxic effect on the physiological and intracellular levels. Indeed, aberrations in LD turnover, such as upregulation of lipolysis can lead to increased levels of circulating fatty acid and result in the development of insulin resistance associated with insulin-dependent diabetes (Saltiel and Kahn, 2001).

Other pathological processes taking place in atherosclerosis and associated with cholesterol homeostasis result first in accumulation of LDs and lipid loaded lysosomes in macrophages, in other words, in transformation of the macrophages to foam cells (Goldstein et al., 1979; Ross, 1995; Small, 1988), and secondly in their apoptotic death (Feng and Tabas, 2002; Yao and Tabas, 2001). Interestingly, intracellular unesterified arachidonic acid also may induce apoptosis (Cao et al., 2000). Furthermore, deposition of unusual fatty acid in LDs and their exclusion from membrane formation might protect the cell from their toxic effect (Dodds, 1995; Lehner and Kuksis, 1996).

These facts raise the question whether the maintenance of intracellular levels of free fatty acids and cholesterol (or other metabolites) is a housekeeping function of LDs, and, as a consequence, whether the basic machinery required for LDs biogenesis is present in all cells of the human body, tightly regulated and used under certain physiological conditions. Several studies provide evidence in favor of this idea. Firstly, all mammalian cells in culture form a number of LDs then grown in medium supplemented with sufficient amounts of fatty acids ((Murphy, 2001) and personal observation). In addition to these *in vitro* observations, supporting evidence may result from the analysis of lipid metabolism aberrations such as human neutral lipid storage disease (NLSD). It is known that NLSD is caused by a breakdown in the regulation of the membrane phospholipid metabolism (Igal and Coleman, 1996; Igal and Coleman, 1998) resulting in accumulation of large numbers of LDs in cells of virtually all tissues examined (Chanarin et al., 1975).

In this context it is worth to note that adipophilin, one of the two most extensively studied LD associated proteins is expressed ubiquitously (Brasaemle et al., 1997b; Heid et al., 1998), however the minimum set of proteins required for LDs biogenesis remains to be identified.

1.1.2 LD biogenesis and turnover

The current model of LD biogenesis in mammalian cells was described in detail by Murphy (2001). The author proposed a constitutive cycle of LD formation and turnover, which may operate in all cells and proceeds as follows:

1) nutrients such as fatty acids are imported into the cell and transported to the ER;
2) in specific regions of the ER where triacylglycerol (or other neutral lipids) biosynthetic enzymes may be clustered, neutral lipids are synthesized and accumulate between the ER membrane leaflets;
3) Nascent LDs are released into the cytoplasm;
4) LD maturation or, in other words, their increase in size, occurs via coalescence of preexisting LDs; if remodeling of the LD content takes place, it occurs via fusion this the ER membrane;
5) Storage of the neutral lipids: in case of adipocytes, LD turnover is slowed down or blocked in favor of the storage mode by perilipins;
6) Recycling/utilization: in case of adipocytes involves hormonal stimulation.

To explain the model in more detail we will focus on processes leading to accumulation of triacylglycerol.

Unesterified long chain fatty acids used for triacylglycerol synthesis are mainly imported into the cells and derive from plasma lipoproteins, i.e. chylomicrones and very low density lipoproteins (VLDL), or fatty acids bound to serum albumin (Murphy, 2001). The mechanism of fatty acid uptake is not entirely clear yet, however, it exhibits many of the kinetic properties of a facilitated process and is assumed to be predominantly transporter mediated (Gao and Serrero, 1999; Stahl et al., 2002). At least three membrane-associated fatty acid-binding proteins were proposed to play a role in fatty acid uptake: plasma membrane fatty acid binding protein (FABPpm), fatty acid translocase (FAT) and fatty acid transport protein (FATP) (Luiken et al., 1999). According to the model proposed by Stremmel and coworkers (2001), the fist step in the fatty acid uptake is their dissociation from serum albumin, involving FABPpm or FAT. Then, fatty acids can flip-flop from the outer membrane leaflet across the bilayer and associate with caveolin-1 or the small molecular weight cytosolic FABPs, a family of tissue specific proteins involved in shuttling fatty acids to cellular

compartments, modulating intracellular lipid metabolism, and regulating gene expression (Boord et al., 2002).

Cellular uptake of long chain fatty acids can be a subject for hormonal regulation, since it was shown that translocation of the fatty acid transport proteins 1, 4 to the plasma membrane and the fatty acid uptake can be induced by insulin or inhibited by TNF-α in adipocytes (Stahl et al., 2002).

Metabolisation of fatty acids requires the conversion to their CoA esters, a reaction that can be performed by FATP1-5/6 (Stremmel et al., 2001) or fatty acid (long chain) CoA ligase (FACL) also known as acyl-CoA synthetase (ACS) and precedes both triacylglycerol synthesis or β-oxidation (Fujino et al., 1996).

Triacylglycerols, which are the major components of LDs in most cell types, are synthesized from acyl-CoA and a product of glucose catabolism, which can be either glycerol 3-phosphate (predominant pathway) or dihydroxyacetone phosphate (minor pathway). The minimal set of enzymes for triacylglycerol synthesis from glycerol-3-phosphate would include:

1) acyl-CoA synthetase to convert fatty acids into fatty acyl-CoA esters;
2) Glycerol-3-phosphate acyltransferase, which esterifies the *sn*-1 position of glycerol-3-phosphate using acyl-CoA to gain lysophosphatidic acid;
3) Lysophosphatidic acid acyltransferase or 1-acyl-glycerol-3-phosphate acyltransferase to synthesize phosphatidic acid;
4) Phosphatidic acid phosphatase, which hydrolyse phosphatidic acid to yield diacylglycerol;
5) Acyl-CoA-diacylglycerol acyltransferase catalyzing the last steps in the triacylglycerol synthesis (Sorger and Daum, 2003; Vance, 1998).

The localisation of most neutral lipid synthesizing enzymes in mammalian cells remains uncertain (Buhman et al., 2001), however, usually it is assumed that triglyceride synthesis takes place in the ER (Cases et al., 1998) and it has been speculated that the enzymes may be clustered in special regions of the ER that are sites of LDs formation and release (Gibbons et al., 2000). This issue has been much better studied in yeast and resulted in a different model for LDs biogenesis (see discussion).

According to the current model for LD formation in mammals, described above, newly synthesized triacylglycerols accumulate between the leaflets of the ER membrane

(Ostermeyer et al., 2001) and eventually bud off or stay in intimate connection with the ER. Perhaps, the origination of LDs from the ER was already visualized in live cells transfected with GFP-tagged mutant caveolin-3. Caveolins are proteins known to be associated with LDs under natural (Fujimoto et al., 2001; Liu et al., 2003) or experimental conditions, when wild type or munant forms of caveolin can be artificially enriched in the ER and redirected to the LD surface (Ostermeyer et al., 2001; Pol et al., 2001). Pol and coworkers (2001) documented the formation of structures containing a caveolin-3 mutant emerging from special regions of the ER, however, the presence of neutral lipids in the observed newly formed structures was not shown.

Indeed, LDs might originate from the ER and stay in intimate connection with it while the areas of continuity between the surface layer of LDs and the membrane leaflets of the ER were revealed by electron microscopy (Blanchette-Mackie et al., 1995) and while most of YFP- or GFP-adipophilin containing structures were found in association with the ER in live HuH-7 and Vero cells (Targett-Adams et al., 2003).

In case of budding of nascent LDs from the ER, further maturation of the organelles proceeds via multiple cycles of LD fusion and substitution of their coat proteins. Events such as fusion and fission of membrane bound structures imply the presence on LD surface of proteins facilitating recognition, docking and other accompanying phenomena. In fact, during maturation of LDs in adipocytes both adipophilin and S3-12 covering early LDs are substituted for perilipin A (Brasaemle et al., 1997b; Wolins et al., 2003), which is known to affect the distribution of LDs and promotes their clustering required for formation of one giant LD in adipocytes (Brasaemle et al., 2000b). Beside our knowledge about ability of the perilipin A to promote LD aggregation, a first step was made to identify proteins promoting LD fusion and fission. Very recently, Liu et al., (2003) could identify multiple rab proteins (e.g. rab18), the mammalian ortholog of Sec22 and α-SNAP on LDs isolated from CHO cells (Liu et al., 2003).

Fusions of LDs should result in generation of excess membrane relative to volume, which could be corrected via phospholipid hydrolysis by a phospholipase A2 (van Meer, 2001) found in association with the LD in animal (Yu et al., 1998) and plant cells (May et al., 1998).

Upon accumulation in LDs, neutral lipids are constitutively lipolysed and resynthesized what show the experiments on hepatocytes, with the estimated rate of

turnover >1.4 pools/24h (Wiggins and Gibbons, 1992) and even in adipocytes, however, in the last the turnover cycle was slowed down (Gibbons and Wiggins, 1995) by perilipin A (see 1.1.3.1). The best studied mechanism of LD turnover is hormone induced lipolysis in adipocytes and adrenocortical cells, where treatment with adrenalin (Gibbons et al., 2000) or adrenocorticotrophic hormone (Fong and Wang, 1997), respectively, results in PKA-mediated phosphorylation of perilipin A and hormone sensitive lipase (HSL), translocation of HSL to the LD surface, decapsulation of LDs (LD-specific capsule in more detail in 1.1.3.6) and translocation of the storage organelles to the vicinity of mitochondria (the last two steps were demonstrated in adrenocortical cells) (Fong and Wang, 1997).
In contrast to adipocytes and steroidogenic cells, in hepatocytes the overall rate of lipolysis of intracellular triacylglycerol appears to be independent of hormones (insulin and glucagon) (Wiggins and Gibbons, 1992) and involves other lipase(s), probably localised in the ER (Gibbons et al., 2000). Indeed, other lipase(s) might be involved in LD turnover, while HSL-knockout mice retain 40% of wild type levels of triacylglycerol lipase activity even in their white adipose tissue (Osuga et al., 2000; Saltiel, 2000).

In summary, the current model of LDs biogenesis in mammals implies a constitutive cycle of neutral lipid storage and turnover which may operate in all cells, their regulation on both organism and cellular levels, import of the bulk fatty acids from the extracellular environment and their primary incorporation into triacylglycerol by the ER localized enzymes.

1.1.3 Lipid droplet associated proteins

Numerous proteins can be found on the surface of LDs. Recently, several attempts aimed at the proteomic analysis of LD derived from yeasts (Athenstaedt et al., 1999) and mammals (Liu et al., 2003; Wu et al., 2000). Unlike most enzymes, the various structural LD associated proteins in different organisms share no obvious homology with each other. Therefore, and for clarity the following text includes the knowledge about neutral lipid biosynthetic enzymes localised on LDs from various organisms, but focus on structural LD proteins in mammals. The three major mammalian LD associated proteins are Perilipin, ADRP and TIP47 constitute the PAT family. In addition to the PAT proteins, the capsular protein P_{200}, as well as lipotransin were

also characterized as LD associated proteins (reviewed by (Londos et al., 1999)). However, as already mentioned above, besides structural proteins and cytoskeletal elements, various enzymes and signaling molecules can be constituents of LDs.

1.1.3.1 Proteins of PAT family

The most abundant proteins associated with LDs in mammalian cells are perilipin, ADRP and TIP47 as well as more distantly related protein S3-12.

Perilipins

Perilipins are structural LD associated proteins unique for adipose and steroidogenic cells (Blanchette-Mackie et al., 1995; Greenberg et al., 1991; Servetnick et al., 1995). Alternative splicing and polyadenilation of the perilipin mRNA yield four protein isoforms: perilipin A (57 kDa), B (46 kDa), C (38 kDa) and D (26 kDa) containing common N-terminal but distinct C-terminal regions (Lu et al., 2001). The perilipins differ in their tissue distribution, with perilipin A and B being expressed in both adipocytes and steroidogenic cells, while perilipin C and D are found in steroidogenic cells only.

The levels of perilipin A and C are regulated post-translationally and are exclusively associated with LDs (Brasaemle et al., 1997a; Greenberg et al., 1993) where they substitute adipophilin (Brasaemle et al., 1997b) and S3-12 (Wolins et al., 2003) in the process of organelle maturation.

The function of Perilipin A as a lipolytic barrier is controlled by hormonal stimulation (Tansey et al., 2003). It is known that adipocytes and steroidogenic cells share common mechanism for lipolysis of stored neutral lipids that is mediated by protein kinase A (PKA) and HSL. In the mentioned cell types, perilipins are associated with the periphery of the LDs (Blanchette-Mackie et al., 1995; Greenberg et al., 1991). Upon hormonal stimulation perilipin A is hyper-phosphorylated (Clifford et al., 2000; Egan et al., 1990; Greenberg et al., 1991) and coincides with fission of the preexisting large LDs into multiple smaller ones (Clifford et al., 2000; Souza et al., 1998). At the same time HSL translocates to the LD surface from the cytosol (Brasaemle et al., 2000a; Clifford et al., 2000; Egan et al., 1992).

The view of the perilipins as a barrier for lypolisis is further supported by experiments using ectopic expression of Perilipin A or B in fibroblasts which leads to an increase of TAG storage that is resistant to hydrolysis (Brasaemle et al., 2000b). The absence

of the barrier in mice lacking perilipin results in a decrease in adipose mass and elevated basal lipolysis (Martinez-Botas et al., 2000; Tansey et al., 2001).

Adipophilin

Adipose differentiation related protein (ADRP or adipophilin) was first identified in mouse as a protein which mRNA level increases 50-100 fold soon after onset of adipose differentiation (Jiang et al., 1992). The murine ortholog of adipophilin is a 426 amino acid protein, localized to the LD fraction after subcellular fractionation (Wolins et al., 2001). In contrast to perilipins, adipophilin appears to be expressed ubiquitously (Brasaemle et al., 1997b; Heid et al., 1998) and its expression is transcriptionally stimulated by long chain fatty acids (Gao et al., 2000).

The function of ADRP is less well understood than that of perilipin A. First, it was proposed that ADRP may be involved in the import of fatty acids, since overexpression of ADRP in Cos-7 cells leads to an increased uptake of long chain fatty acids either saturated or unsaturated (Gao and Serrero, 1999; Gao et al., 2000). In addition, ADRP interacts with long chain fatty acids in vitro (Serrero et al., 2000). However, ADRP was found exclusively on the surface of LDs (Brasaemle et al., 1997b; Wolins et al., 2001) and does not rapidly diffuse in live cells assuming only a little turnover of ADRP at the surface of LDs after their formation (Targett-Adams et al., 2003). The last two findings suggest that the effect on the fatty acid uptake is indirect and is a kind of positive feedback.

Secondly, the ability of adipophilin to bind a cholesterol analog in vitro was interpreted as a probable participation of the protein in specific targeting of unesterified cholesterol to LDs (Frolov et al., 2000).

Alternatively, it was suggested that ADRP also may act as a lipolytic barrier around lipid droplets (Wolins et al., 2003), since overexpression of ADRP in cultured cells leads to an increase in LD size and number (Imamura et al., 2002) and to elevated fatty acid uptake (Gao and Serrero, 1999).

TIP47

The Tail interacting protein of 47kD (TIP47), also known as a variant of placental protein pp17b, is ubiquitously expressed (Than et al., 1998) and shares 43% of identity with adipophilin. TIP47 was mainly studied as a cargo selection device for mannose-6-phosphate receptors (Barbero et al., 2002; Carroll et al., 2001; Diaz and

Pfeffer, 1998; Hanna et al., 2002; Krise et al., 2000; Orsel et al., 2000; Sincock et al., 2003) (described in detail in 1.2.). Nevertheless, in apparent conflict with the set of data, TIP47 was found in association with LDs (Miura et al., 2002; Than et al., 2003; Wolins et al., 2001), but was not extensively studied. In the very first article on TIP47 as a LD associated protein it was shown that TIP47 localizes to the surface of LDs by indirect immunofluorescence and subcellular fractionation (Wolins et al., 2001). The authors also demonstrated that TIP47 subcellular distribution depends on the nutritional status of cells and that the protein is redistributed to the LD concurrently with triacylglycerol accumulation (Wolins et al., 2001). However, their work was immediately opposed by the group of S.R.Pfeffer (Barbero et al., 2001) who originally characterized TIP47 as a cargo selection device. The authors attributed the discrepancy in the data obtained to cross-reactivity of the used antiserum against TIP47 and insisted that the protein is not a constituent of LDs and localizes to endosomes. Nevertheless, in the next publication on the topic it was shown that GFP-tagged TIP47 concentrates around isolated LDs (Miura et al., 2002) and, later, that endogenous TIP47 surrounds LDs using a different batch of extensively tested antibodies (Than et al., 2003).

S3-12

The protein S3-12 is also related to the PAT proteins and was first identified as a protein induced during adipocyte differentiation (Scherer et al., 1998). In fact, expression of S3-12 is restricted to adipose tissue with higher protein levels in white than in brown fat (Wolins et al., 2003). Interestingly, LD association of S3-12 depends on the nutritional status of the cell, is fully reversible and may require the incorporation of fatty acids into triacylglycerol (Wolins et al., 2003).

1.1.3.2 Neutral lipid biosynthetic enzymes and fatty acid binding proteins

Most of the proteins, found in association with LDs during proteomics studies, are, indeed, various enzymes of lipid metabolism (Athenstaedt et al., 1999; Liu et al., 2003).

In mammals, the first discovered enzymes in LDs belong to the pathway of arachidonate oxidation to eicosanoids and other oxylipid mediators. The enzymes were found on LDs mainly in cells involved in inflammatory response, such as eosinophils and neutrophils. Among the proteins are prostaglandin endoperoxide

synthase (PGH synthase), which is the initial ratelimiting enzyme involved in oxidation of arachidonic acid to produce prostaglandins (Dvorak et al., 1994) and others, including 5-lipoxygenase (5-LOX), 15-LOX and leukotrien C_4 synthase (Bozza et al., 1997; Weller et al., 1999).

Despite the identification of all the principal enzymes involved in arachidonate oxidation, most enzymes of neutral lipids, in particular triacylglycerol, biosynthetic enzymes, which could act on LDs in mammalian cells are still unknown. However, prerequisites for independent of the ER accretion of LDs exist, since, firstly, Wu et al. (2000) demonstrated that LDs isolated from both liver and mammary gland contain fatty acid binding protein (FABP), with could mediate delivery of fatty acids to the LDs (Mather, 2000). Secondly, Liu et al. (2003) found first enzymes required for triacylglycerol synthesis, namely: fatty acid (long chain) CoA ligase 3 and 4, together with 11 other enzymes of the lipid metabolism on the LDs. Our poor knowledge about LD associated triacylglycerol biosynthetic enzymes of mammals can be illustrated by the example of Acyl-CoA-diacylglycerol acyltransferase (DGAT). DGAT, which catalyzes the last reaction in triacylglycerol synthesis, has been purified from the LDs of fungi (Kamisaka et al., 1997). A DGAT, specific to LDs, was characterized in yeast (Sorger and Daum, 2002), however, the enzyme still was not found on LDs derived from mammalian cells (see Table 1.1.).

Interestingly, LDs of an oleaginous fungus, studied with respect of activities of key triacylglycerol biosynthetic enzymes, contained activities of them all (Pillai et al., 1998). Similarly, most of the enzymes were identified in yeast LDs (reviewed in (Sorger and Daum, 2003)) (see Table 1.1.).

Fatty acid transport proteins/ TAG biosynthetic enzymes	Mammalian LDs	Fungus LDs enzymatic activity	Yeast LDs
Fatty acid transport protein	cFABP		FAT1
Fatty acid-CoA ligase	ACS/FACL3,4		FAA1,4
Glycerol-3-phosphate acyltransferase	-	+	Gat1p
1-acyl-glycerol-3-phosphate acyltransferase	-	+	SLC1[c]

Phosphatidic acid phosphatase	-	+	-
Acyl-CoA-diacylglycerol acyltransferase	-	+	Dga1p

Table 1.1. Fatty acid transport proteins and enzyme of triacylglycerol biosynthesis of glycerol-3-phosphate pathway found on LD in yeast and mammals.

Numerous enzymes were identified as constituents of LDs, however, some of the key enzymes of various biosynthetic pathways are still missing, others are inactive when localize to LDs, i.e. squalene epoxidase (Leber et al., 1998) and NAD(P)H steroid dehydrogenase-like protein (Ohashi et al., 2003). Earlier the presence of the biosynthetic enzymes on LDs was called to question (Murphy, 2001), however, the localization was demonstrated already in several organisms and by multiple techniques, and what is, probably, more important, LD specific triacylglycerol and cholesterol biosynthetic enzymes were identified. The enzymes, which almost exclusively localize to LD in yeast, are oxidosqualene cyclase (Milla et al., 2002) and DGAT - Dga1p (Sorger and Daum, 2002). The last makes the LDs one of the two major sites of triaclyglycerol synthesis in yeast (Sorger and Daum, 2003).

1.1.3.3 Lipolytic enzymes

As many other aspects of LD biology the exact mechanisms of LD turnover are not entirely understood yet. However, the number of publications describing LD associated lipolytic enzymes is growing. The best studied among the enzymes is a hormone sensitive lipase (HSL). HSL mediates hormone stimulated mobilization of neutral lipids stored in adipocytes and steroidogenic cells. HSL is a cytoplasmic protein, which transiently associates with LD surface after phosphorylation by PKA (Clifford et al., 2000; Egan et al., 1992). The lipase can be bound to the organelle membrane via a docking protein - lipotransin identified as structural components of adipocytes LDs (Syu and Saltiel, 1999). The HSL is responsible for at least 60% of triacylglycerol lipolysis in the white adipocytes and for all neutral cholesterol ester hydrolase activities in the testes, brown and white adipose tissue (Osuga et al., 2000). At the same time, HSL may participate in LD turnover in cardiomyocytes and pancreatic β-cells (Gibbons et al., 2000).

In addition to the well known HSL, another tissue specific enzyme was found on LDs is a neutral triacylglycerol hydrolase (TGH) present as well in microsomal fractions from liver, kidney and intestine (Lehner et al., 1999). Nevertheless, findings of the

enzymes can not explain recycling of LDs in other cell types. The candidates for ubiquitous lipolytic enzymes associated with LD are CGI-58 and phospholipase A2.
The first of them, CGI-58, which belongs to the esterase/lipase/thioesterase family of proteins, was identified as a constituent of LDs in CHO K2 cells (Liu et al., 2003). Defects in the CGI-58 gene lead to abnormal accumulation of LDs in cells of multiple tissues, that results in a neutral lipid storage disease (NLSD) (Lefevre et al., 2001).
The second potentially ubiquitous enzyme detected on LDs of both plants (May et al., 1998) and animals (Yu et al., 1998) is phospholipase A2. Noll et al. (Noll et al., 2000) hypothesized that the enzyme may partially degrade the phospholipid monolayer at the onset of triacylglycerol mobilization, thus providing access for other lipases to the neutral lipid core of LD. On the other hand, phospholipase A2 may be responsible for removal of the excess phospholipids from LD surface resulting from the organelle fusions (van Meer, 2001).
In addition, a putative lipase, related to both, plant seed phospholipase A2, and yeast LD associated triacylglycerol lipiase YMR313c, was recently identified as a constituent of LDs isolated from CHO K2 cells (Liu et al., 2003). Notably, on LDs isolated from yeast three more putative lipases are present (Athenstaedt et al., 1999)

Five mammalian lipolytic enzymes mentioned above can at least transiently associate with LDs in various tissues and promote metabolisation of the stored lipids, however, it is not clear yet whether at list some of the lipolytic enzymes are always present on the surface of LDs to mediate the continuous recycling of the neutral lipids, since even CGI-58, that is the candidate for recycling of triacylglycerol in multiple tissue cells, translocates to LD upon induction with oleic acid (Liu et al., 2003).
It is possible, that partial degradation of the phospholipid monolayer by a phospholipase as it was proposed for phospholipase A2 (Noll et al., 2000) may precede hydrolysis of neutral lipids by the other lipolytic enzymes.
The growing knowledge about constituents of LDs suggests that turnover of the organelles, in particular, hydrolysis of the stored lipids, may not require association with other metabolic organelles, e.g. the ER.

1.1.3.4 Reticuloplasmins

Beside the various enzymes of lipid metabolism, LDs were reported to contain several ER proteins (some of them are found in the cytoplasm as well) important for proper protein folding, such as the molecular chaperones immunoglobulin binding protein (BiP) (Ghosal et al., 1994; Prattes et al., 2000), calreticulin (Ghosal et al., 1994) and calnexin (Prattes et al., 2000) as well as protein disulfide isomerase (PDI) (Ghosal et al., 1994). In a recent publication, the data were confirmed using biochemical methods on LDs isolated from CHO cells, which contain BiP, PDI, sec61 and heat shock protein 71 (HSP71). However, the last two proteins were regarded as contaminants (Liu et al., 2003) and it still remains to be clarified to which extend ER proteins localise to LDs or whether they are constituents of LDs at all.

1.1.3.5 Membrane traffic related proteins

LDs are membrane bound organelles, which undergo fusion and fission, however, the underlying mechanisms and proteins facilitating the processes remain obscure. It was proposed that the cytoskeleton could control LD dynamic (Murphy, 2001), but the idea was not entirely developed yet. In the most recent publication, Liu et al. (2003) could identify several proteins known to be involved in membrane traffic associated with LD, in particular, nine rab proteins (e.g. rab18), which are the small GTP-ases regulating docking and fusion of membrane-bound organelles (Soldati et al., 1995), the mammalian ortholog of Sec22, one of the ER resident SNARE proteins (soluble NSF attachment protein receptor) (Hay et al., 1996), α-SNAP (α-soluble NSF attachment protein), which functions in dissociation of the SNARE complexes (Marz et al., 2003), as well as RalA, a small GTPase regulating, among other processes, membrane traffic in polarized cells (Moskalenko et al., 2002).
In case these findings will be confirmed, that would support the idea that membrane trafficking of organelles bound with a single or double leaflet membrane share common features.

1.1.3.6 Cytoskeleton

The association of LDs with the cytoskeleton was demonstrated by means of light and electron microscopy (Franke et al., 1987; Targett-Adams et al., 2003; Wang et al., 1997) and was also partially biochemically characterized. Up to now the most remarkable LD-associated cytoskeletal structures are regular vimentin cages formed during adipose conversion of 3T3-L1 cells (Franke et al., 1987; Lieber and Evans,

1996; Wang et al., 1997). In contrast to adipose, in steroidogenic cells LDs are randomly attached to vimentin filaments (Almahbobi and Hall, 1990; Almahbobi et al., 1992). Despite the different organisation of vimentin cytoskeleton, in both cell types LDs are associated with a capsular protein P_{200} (Fong and Wang, 1997; Fong et al., 1996; Wang and Fong, 1995; Wang et al., 1997), which was supposed to function as a linker between the LD membrane and vimentin filaments. Indeed, P_{200} could promote rearrangement of vimentin, since P_{200} translocation to the nascent LDs follows the adipose conversion of preadipocytes and precedes the vimentin cage formation (Lieber and Evans, 1996; Wang et al., 1997). Moreover, P_{200} may serve as a regulator of the association with cytoskeletal elements, since it detaches from LDs and translocates to the cytosol upon hormonal stimulation of steroid secretion (Fong et al., 2002; Wang and Fong, 1995).

LDs in other cells types are not surrounded with vimentin cages or P_{200}, but may interact with microtubules and/or actin cytoskeleton. The transport of LDs from the basal region to the apical membrane of mammary epithelial cells is supposed to be necessary for milk lipid globule secretion (Wu et al., 2000). Attempts to elucidate the components of the transport machinery associated with LDs also revealed the presence of at least four potentially important proteins, including dynein intermediate chain (facilitating interaction of cargo with microtubules), gelsolin (implicated in actin remodeling), gephyrin (involved in membrane protein-cytoskeleton interaction) and motor protein (of unknown function) (Wu et al., 2000).

The vectorial transport (most likely along microtubules) of a minor fraction of LDs was visualized in HuH-7 cells (Targett-Adams et al., 2003).

Thus, LDs could be integrated into the intracellular environment by means of various cytoskeletal elements: vimentin, actin and microtubules, in a tissue specific manner.

1.1.4 LDs are complex metabolically active organelles

There is growing evidence in favour of the idea that LDs are complex organelles directly participating in both lipid metabolism and intracellular signalling since they possess multiple proteins which could be potentially involved in the processes. Moreover, the most recent publications (Athenstaedt et al., 1999; Liu et al., 2003; Sorger and Daum, 2003), describing LD associated triacylglycerol biosynthetic enzymes and lipases, support the hypothesis that LDs have the potential of independent from other organelles neutral lipids biosynthesis and turnover.

1.2 Transport of mannose 6-phosphate receptors and TIP47

Vesicle-mediated transport is a process required to establish and to maintain the unique composition of eukaryotic membranous organelles. Organelles of the secretory and endocytic pathways such as the endoplasmic reticulum (ER), the Golgi apparatus (GA), the trans-Golgi network (TGN), as well as endosomes and lysosomes continuously exchange material with each other. The process includes formation of a transport intermediate or a vesicle and its fusion with an acceptor compartment. Selection of cargo proteins into a newly forming vesicle requires a sorting event during which a specific interaction between the cargo proteins and cargo selection devices or adaptor molecules which are part of the transport machinery occurs. This machinery includes scaffolding proteins, such as clathrin, multiple GTPases, regulating docking and fusion of membrane-bound organelles, motor proteins, such as dynamin, SNAREs and other proteins promoting docking and fusion with an acceptor membrane.

Two of the model cargo proteins whose transport was intensively studied so far are the mannose 6-phosphate receptors (MPRs). The MPRs are transmembrane proteins continuously recycling in the secretory and endocytic pathways (see Figure 1.1.). The function of the receptors is binding and delivery of soluble lysosomal enzymes to late endosomes. There are two MPRs different in molecular weight and calcium dependency called MPR46 (calcium-dependent) and MPR300 (calcium-independent). Both receptors have similar transport routes in the cell.

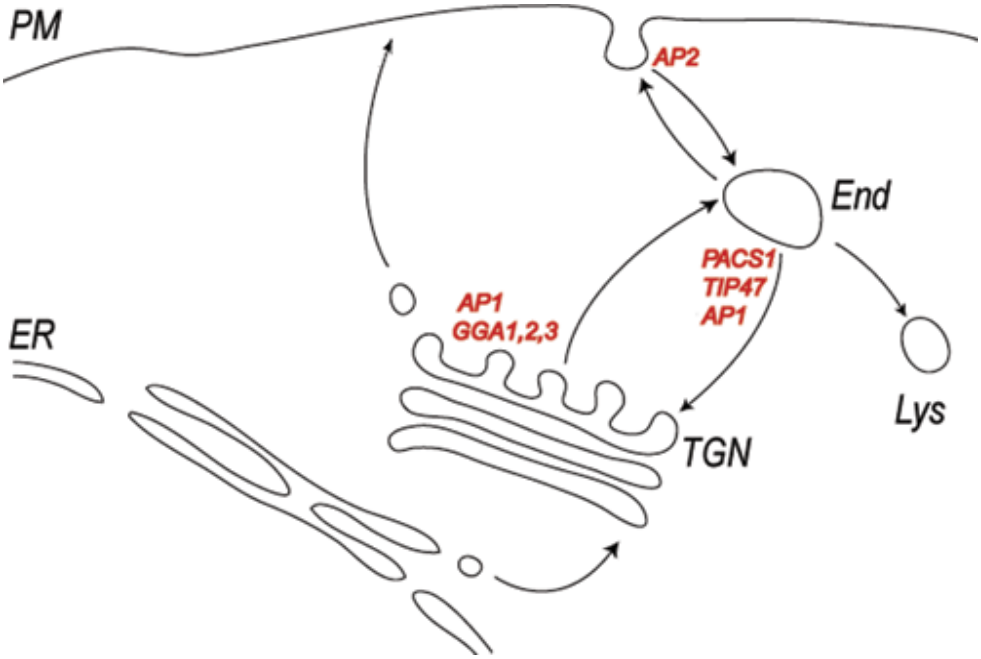

Figure 1.1. Transport route of MPRs in the cells. Newly synthesized MPRs are transported from the ER through the Golgi stack to the TGN where they are recruited into clathrin coated pits by means of adaptor complex AP1 and GGA1, 2, 3 proteins and delivered to an endosomal compartment (End). Alternatively, MPRs escape to the plasma membrane (PM) and are internalized from the cells surface by means of adaptor complex AP2 (clathrin coated vesicles). From the endosomes MPRs are either retrieved to the TGN (candidate cargo selection devices are AP1,3, PACS1 or TIP47) or finally degrade in the lysosomes (Lys).

Adaptors involved in MPRs transport from various organelles are AP1 and GGAs (Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding proteins) at the TGN and AP2 at the plasma membrane. AP1 (Mallard et al., 1998; Meyer et al., 2000), AP3 (Medigeshi and Schu, 2003; Storch and Braulke, 2001), PACS1 (Wan et al., 1998) and TIP47 (Diaz and Pfeffer, 1998) are other candidates which could determine the retrieval of MPRs from an endosomal compartment to the TGN.

The view on TIP47 as a cargo selection device was based on a row of evidences, which seems to be clear. TIP47 was initially identified in a yeast two-hybrid system as a protein interacting with MPRs cytoplasmic domains (Diaz and Pfeffer, 1998). Further analysis of the protein revealed that it binds MPRs cytoplasmic domains expressed and purified as GST fusion proteins and colocalizes with MPR300 in endosomes (Diaz and Pfeffer, 1998). Moreover, the authors claimed that TIP47 association with membranous organelles requires the presence of MPRs and is GTP-ase stimulated. In the same work TIP47 was found to be important for recycling of MPRs from the endosomes to the TGN in an in vitro transport assay and in live cells, where cytosolic amounts of TIP47 were decreased by antisense oligonucleotides. Later, the interactions of TIP47, MPR46, MPR300 and the small GTP-ase, which

turned out to be rab9, were studied in detail (Carroll et al., 2001; Diaz and Pfeffer, 1998; Hanna et al., 2002; Krise et al., 2000; Orsel et al., 2000; Sincock et al., 2003).

Summarizing, S.R.Pfeffer and colleagues postulated that TIP47 localizes on endosomes and is required for MPRs recycling in vivo and in vitro and that the sorting event is facilitated by a small GTP-ase rab9.

2 Aim of the study

The trafficking of the mannose 6-phosphate receptors (MPRs) has been studied for many years, but still some important details remain controversial. For example, it is still an ongoing debate how MPRs are sorted for recycling from endosomes to the TGN. Several adaptor molecules have been postulated to play an important role in the process including AP1 and TIP47. Mainly due to the work of Suzanne Pfeffer and colleagues, TIP47 was characterized as a cargo selection device for MPRs, determining their transport from endosomes back to the TGN. However, several other groups recently found TIP47 associated with the surface of lipid droplets and suggested that TIP47 functions in lipid metabolism. Nevertheless, a direct functional proof for this concept is still missing. Lipid droplets are peculiar storage organelles and supposed not to have any relationship with MPR trafficking pathways. Due to these uncertainties about the functional role of TIP47, the aim of this study was:

1. to revise the intracellular TIP47 localization and its recruitment to membranes using endogenous protein as well as cells expressing recombinant TIP47.
2. to establish a cell line not expressing TIP47 by a plasmid based RNA interference technique.
3. to study MPR46 distribution and recycling in such TIP47 deficient cells.
4. to analyze the putative function of TIP47 in lipid metabolism/lipid droplet formation.

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3 Materials and Methods

3.1 Materials

3.1.1 Equipment used for molecular biology and biochemical work

Analytical weighing balance Type CP225D & M5P	Sartorius, Göttingen
Autoclave Type Tecnoclav 50	Tecnorama, Zürich, Switzerland
Balances Type BP3100P & CP3202P	Sartorius, Göttingen
Chamber for PAGE and wet Western blotting Mini-V 8x10	Gibco BRL Life Technologies, Inc
ELISA reader, TECAN SPECTRA	SLT Instruments, Germany
Electrophoresis Power Supplies: Biometra P25 & PP4000 St 305	 Schüt Labortechnik, Göttingen Gibco BRL Life Technologies, Inc
Electroporator 1000 Stratagene®	Cortland NY, USA
Refrigeretor +4°C	Liebherr
Freezer –20°C	Liebherr
–85°C Ultra Low Freezer	New Brunswick Scientific Co, Inc., Edison, USA
Gel documentation system	Hitachi, Japan
GelAir Dryer	Bio-Rad, USA
Heating block 5320 & 5436	Eppendorf, Hamburg
Ice Machine	Ziegra, Isernhagen
Incubator for bacterial liquid cultures	New Brunswick Scientific Co, Inc., Edison, USA
Incubator for bacterial plates	Heraeus, Osterode
Incubators for mammalian cells: Infrared CO_2 incubator Labotec	 Labotect GmbH, Göttingen

STERI:CULT incubator	Forma Scientific, Ohio
Liquid Nitrogen tanks Biosafe Chronos	Messer Griesheim, Frankfurt/M
Liquid Scintillation Counter LS 6500	 Beckman, USA
Luminescent Image Analyzer Fujifilm LAS-1000 plus	Fuji photo film Co. Ltd., Japan
Magnetic stirrers: Ikamag Ret MR3000	 Janke & Kunkel, Staufen Heidolph, Germany
Microflow Biological Safety Cabinet	Nunc, Wiesbaden
SterilGARD Hood	the BAKER COMPANY, inc Sanford, Maine
Microwave Oven	Siemens, Germany
pH-Meters: Knick ino Lab pH Level 1	 Schütt, Göttingen WTW, Weilheim
Phosphoimager Fujix BAS1000	Fuji, Tokyo, Japan
Pipet-aid	Drummond Scientific Corporation, USA
RNA/DNA Calculator Genequant II	Pharmacia Biotech Cambridge, England
Rocker	Institute workshop
Sonicators:	
Sonicator™ W-200F	Heat systems-Ultrasonics, INC New York, USA
Sonifier 450	Branson Ultrasonic SA, Carouge Geneva/ USA
Spectrophotometer, Uvikon 932	Kontron Instruments, Italy
Speed Vac Concentrator	BACHOFER, Germany
Spinning Wheel	Institute workshop

Thermocycler Mastercycler Gradient	Eppendorf, Hamburg
Transilluminator IL-400-M	Bachofer, Reutlingen
UV-hand lamp (254nm and 312nm)	Bachofer, Reutlingen
Vortex Genie 2 Vortex Genie 2^{TM}	Bender & Hobein AG Zurich, Switzerland
Water bath Julabo U3	Julabo Labortechnik GMBL
Water bath shaker Köttermann	Ernst Schütt Jr, Göttingen

Centrifuges

Labofuge GL MC13 Megafuge 1.0	Heraeus Sepatech, Osterode/ Harz
Eppendorf centrifuges: 5415C, 5415D & 5417R	Eppendorf, Hamburg
Microprep centrifuge StartSpin µPrep	Schütt Labortechnik, Göttingen
Refrigerated centrifuge J2-MC Ultracentrifuge L-80 Ultracentrifuge L8-70M	Beckman, München
Rotors for cooling centrifuges and ultracentrifuge: (g values are for r_{max})	
JA-10 rotor, upto 17,680 g	Beckman, München
JA-20 rotor, upto 48,300 g	Beckman, München
SW41Ti rotor, upto 210,053g	Beckman, München

3.1.2 Equipment used for microscopy

Flourescence Microscope Axiovert 100	Zeiss; Oberkochen
Phase-contrast microscope Model ID 03	
Laser Scanning Microscope LSM2 with software 3.95	
Objectives for the LSM and Axiovert 100 Plan-Neofluar 25x/0,8 1mm Plan-Neofluar 40x/1,3mm Plan-Neofluar 63x/1,25mm Plan-Neofluar 100x/1,3mm Plan Apochromat 63x/1,4mm	Zeiss; Oberkochen
Leica TCS SP2 AOBS Filter-free microscope	Leica Microsystems Heidelberg GmbH, Germany
Objectives for Leica DM IRE2 HC PL APO 20x/0.70 HCX PL APO 40x/1.25 HCX PL APO63x/1.40-0.60	Leica Microsystems Heidelberg GmbH, Germany
Olympus IX50 Fluorescence microscope	Olympus Optical Co., LTD Japan
Objectives for the Olympus IX50 UPlan Apo 40x/1,00mm Plan Apo 60x/1,40mm UPlan Apo 100x/1,35mm	Olympus Optical Co., LTD Japan
Olympus TH4-200	Olympus Optical Co., LTD Japan
CCD Camera Imago ½" chip with 640x480 pixels	TILL Photonics GmbH
Monochromator Polychrome II	TILL Photonics GmbH

3.1.3 Materials for molecular biology and biochemical experiments

Autoclave bags	Sarstedt , Nümbrecht
Bottle top filters	Sarstedt Inc., Newton, USA
Cell Culture single use pipettes 5ml & 10ml	Greiner, Nürtingen
Cell Culture plates:	
4 well NUNCLON	Nalge Nunc International Denmark
6 well, 24 well & 96well CELLSTAR	Greiner bio-one, Frickenhausen
Centrifuge tubes:	
JA-10 Polypropylene tube	Nalgene, München
JA-20 Polypropylene tube	Nalgene, München
14x89mm Polyallomer tube	Beckman Instruments, Inc., USA
Culture dishes for mammalian cells	
3; 5; 6; 10 & 15 cm Ø	Greiner bio-one, Frickenhausen
Culture flasks for bacteria	Schott, Mainz
Culture flasks for mammalian cells	
25 and 75 cm^2	Greiner bio-one, Frickenhausen
Dialysis bags (12,000 Da cut-off)	Biomol, Hamburg
Delta T dishes 0.15mm	Bioptechs, Inc., USA
Glass pipettes	Schütt, Göttingen
Immersion oil 518C	Zeiss, Oberkochen
Microscope slides	Menzel-Glaser
Needles for syringes	B.Braun Melsungen AG, Melsungen
Nunc cryotube™ vials	Nalge Nunc International Denmark
Parafilm	American National Can™ Chicago
Pasteur pipettes	Schütt, Göttingen
Pipette tips	Sarstedt, Nümbrecht
Plastic tubes 10, 15 and 50 ml	Sarstedt, Nümbrecht
Polycarbonate ultracentrifuge tubes 1,5ml	Beckman, München

Reaction tubes:	
0.2, 0.5, 1.5 & 2 ml	Sarstedt, Nümbrecht
Scintillation Cocktail LUMASAFE PLUS	Lumac LSC B.v., The Netherlands
Scintillation Vials MINIS 2000	Zinsser Analytic, Germany
Sterile filters 0.2 µM	Heinemann Labortechnik GmbH
Syringes:	
1 ml and 2 ml	B.Braun Melsungen AG, Melsungen
5, 10 & 20 ml Ecoject	Dispomed Witt OHG, Gelnhausen

3.1.4 Materials for Western Blotting

Nitrocellulose membrane 0.2 µM	Schleicher and Schüll, Dassel
Whatman GB002 paper	Schleicher and Schüll, Dassel
Whatman GB003 paper extra thick	Schleicher and Schüll, Dassel
Supersignal Chemiluminiscent Substrtate	Pierce, Illinois/ USA

3.1.5 Chemicals

Acetic acid	Roth
Acetone	Merck
Acrylamide/ Bisacrylamide (30%/ 0.8%)	Roth
Agar	Roth
Agarose	Roth
Ammonium chloride	Sigma
Ammonium peroxide disulphate (APS)	Merck
Bacto tryptone	Roth
Bio-Gel P-6 DG (Desalting gel)	Bio-rad
Bromophenol blue	Merck
Calcium chloride	Merck
Calcium acetate	Sigma
Chloroform	Merck
Coomassie, Serva Blue R	Serva
DAKO (Fluorescent mounting medium)	Dako Corporation

Dimethylsulfoxide (DMSO)	Merck
Disodiumhydrogenphosphate	Merck
Dipotassiumhydrogenphosphate	Merck
Dithiothreitol (DTT)	Diagnostic Chemicals Ltd
Ethanol	Roth
Ethidium bromide	Serva
Ethylenediamine tetra acetic acid -sodium salt (EDTA)	Merck
Glucose	Merck
Glutathione	Amersham Pharmacia
Glycerol	Roth
Glycine	Roth
HEPES (N-2-Hydroxyethylpiperazin-N'-2-ethanesulfonic acid)	Roth
Isobutanol	Merck
Isopropyl alcohol	Merck
Liquid Nitrogen	Messer Griesheim, Seigen
Magnesium chloride	Merck
Manganese chloride	Merck
2-Mercaptoethanol	Merck
Methanol	Roth
MOPS (3-[N-Morpholino]propanesulfonic acid)	Serva
Nile Red	Sigma
Paraformaldehyde (PFA)	Sigma
Ponceau S (0.2% solution in 3% TCA)	Serva
Potassium chloride	Merck
Potassium hydroxide	Merck
Oleic acid	Sigma
Rubidium chloride	Merck
Silver nitrate	Sigma
Sodium acetate	Merck

Sodium azide	Sigma
Sodium bicarbonate	Merck
Sodium chloride	Roth
Sodium dihydrogenphosphate	Merck
Sodium hydroxide	Merck
Sodium thiosulfate	Sigma
Sucrose	Merck
TEMED (N'N'N'N Tetramethylethylenediamine)	Serva
Trichloroacetic acid	Merck
Triethanolamine	Merck
Tris-base	ICN Biomedicals, Aurora/ USA
Yeast extract	Roth

All the chemicals were of analysis grade.

3.1.6 Detergents

NP-40	Serva
Saponin	Sigma
Sodium deoxycholate	Serva
Sodium dodecyl sulphate (SDS)	Sigma
Triton X-100	Serva
Tween-20	Serva

3.1.7 Enzymes and Nucleotides

Restriction endonucleases	New England Biolabs
T4 DNA ligase	New England Biolabs
Pfu Ultra^{TM} DNA Polymerase	Stratagene
Taq DNA polymerase	Pharmacia, Freiburg
Ultra pure dNTP set	Pharmacia, Freiburg

3.1.8 Proteins, Protease inhibitors and Protein standards

Aprotinin	Biomol
Bovine Serum Albumin (BSA)	Serva
Glutathione-sepharose	Amersham-Pharmacia
Leupeptin	Biomol
PANSORBIN cells, Standardized (Heat inactivated *Staphylococcus aureus* cell suspension)	Calbiochem
Pepstatin A	Biomol
Phenylmethylsulfonylfluoride (PMSF)	Serva
Prestained protein molecular weight standards	Biorad
Protein A agarose	Sigma
Protein G sepharose	Sigma

3.1.9 Vectors and DNA standards

pGK Hygro	Invitrogen life technologies
pMPSV-EH	
pSHH	IMGENEX Corporation
DNA ladder	Gibco BRL

3.1.10 Antibiotics and Drugs

Ampicillin	Serva
Kanamycin	
Neomycin (Gentamycin sulfate or G418)	Gibco
Penicillin/Streptomycin (100 x =10,000 U/ml)	Gibco

3.1.11 Radioactive substances

L-[^{35}S]-Methionin/Cystein, aqueous solution 14mCi/ml

Purchased from Amersham Pharmacia Biotech, UK.

[γ-^{32}P]-ATP 10mCi/ml

Purchased from Hartmann Analytic GmbH, Germany

[1-^{14}C]-Arachidonic acid, 0.050mCi/ml

[1-^{14}C]-Oleic acid, 0.1mCi/ml

[9,10-^{3}H]-Palmitic acid, 1.0mCi/ml

Purchased from Moravek Biochemicals Inc, USA

3.1.12 Kits

ABI PRISM® Rhodamine Terminator	Applied Biosystems
Cycle sequencing Ready Reaction Kit	Perkin Elmer Applied Biosystems
Bio-Rad Protein Assay	Bio-Rad
Effectene Transfection kit	Qiagen
GeneSilencer™ System	IMGENEX Corporation
HiSpeed Plasmid Midi kit	Qiagen
Invisorb Spin Plasmid Mini kit	Invitek, Berlin
QIAquick Gel extraction kit	Qiagen
QuikChange™ Site-Directed mutagenesis kit	Stratagene

3.1.13 Primary Antibodies

Name	Type	Antigen	Immunized Species	Reference
MSC 1	Polyclonal	tail peptide of MPR46	Rabbit	(Klumperman et al., 1993)
KII5	Polyclonal	human MPR46	Rabbit	
γ–adaptin	Monoclonal	Mouse γ–adaptin hinge region	Mouse	Transduction laboratories
α–adaptin	Monoclonal	Mouse α_A-adaptin N – terminus	Mouse	Transduction Laboratories
I 5	Polyclonal	Rat MPR-300	Rabbit	(Claussen et al., 1995)
TIP47	Polyclonal	Synthetic peptide of TIP47 amino terminus aa 1-16	Guinea pig	Progen Biotechnik
TIP47	Affinity purified polyclonal	His-tagged TIP47	Rabbit	(Barbero et al., 2001; Diaz and Pfeffer, 1998)
PDI	Monoclonal	Synthetic peptide residues 499-509	Mouse	StressGen

GM130	Monoclonal	C-terminus of rat protein residues 869-982	Mouse	Transduction Laboratories
HA	Monoclonal, purified	Mono HA-tag	Mouse	Covance
His	Monoclonal	RGS-HHHH	Mouse	Qiagen
Lamp1	Monoclonal	Human lamp1	Mouse	Hybridoma Bank
LDH	Polyclonal		Rabbit	Prof.Dr. Söling, MPI for Biophysical Chemistry, Göttingen
Rab9	Monoclonal	Recombinant canine rab9	Mouse	Affinity Bioreagents, INC

Table 3.1. List of antibodies used in this study.

3.1.14 Secondary antibodies

Goat anti-mouse and goat anti-rabbit antibodies conjugated with Alexa633 were from Molecular Probes, other secondary antibodies listed below were purchased from Dianova, Hamburg.

Donkey anti-goat Texas Red conjugate

Goat anti-guinea pig Flourescein isothiocyanate conjugate

Goat anti-guinea pig Horseradish peroxidase conjugate

Goat anti-mouse Horseradish peroxidase conjugate

Goat anti-rabbit Horseradish peroxidase conjugate

Goat anti-mouse Cy2 conjugate

Goat anti-rabbit Cy2 conjugate

Goat anti-mouse Cy3 conjugate

Goat anti-rabbit Cy3 conjugate

3.1.15 Bacterial strains and mammalian cell lines

Escherichia coli:

Strain	Genotype	Firm
DH5α	supE44, thi-1, recA1, relA1, hsdR17(rK-mK+), thi-1, ΔlacU169 (Φ80 lacZΔM15), endA1, gyrA (Nal^r)	Gibco BRL, Eggenstein
XL1-Blue	recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac [F', proAB, $lacI^qZ\Delta M15$, $Tn10(Tet^r)]^c$	Stratagene, Heidelberg
BL21-(D3)-RIL	*E. coli* B F^- ompT hsdS(r_B^- m_B^-) dcm^+ Tet^r gal λ(DE3) endA Hte [argU ileY leuW Cam^r]	Stratagene, Heidelberg

Mammalian cell lines

Cos 7: green monkey kidney cells

HeLa: human cervical carcinoma cells

HeLa cells stably transfected with TIP47 anti-sense construct (this work)

3.1.16 Media and Solutions for Cell culture

PBS (Sterile for cell culture)	150 mM NaCl 120 mM KCl 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4
Dulbecco's Modified Eagles Medium (DMEM)	Gibco-Invitrogen
Fetal Calf Serum (FCS)	PAA laboratories GmbH, Austria
Trypsin-EDTA solution (1x) 0.5 g/L Trypsin 0.2 g/L EDTA in Puck's Salt Solution A without Ca^{2+} and Mg^{2+}	Gibco
L-Glutamine (100 x) (200 mM)	Gibco
Phosphates, pyruvate-free DMEM	PAN Biotech GmbH
Sulfate-free DMEM powder	PAN Biotech GmbH

3.1.17 Stock solutions and buffers

<u>10 x PBS</u>	100 mM sodium phosphate buffer pH 7.4

	9 % sodium chloride Dissolved in 800 ml water, volume was made up to 1000 ml and autoclaved. Stored at room temperature.
1 x TBS	10 mM Tris/ HCl pH 7,4 150 mM Sodium chloride
1 x TAE	0.04 M Tris-acetate 1mM EDTA (pH 8.0)
50 x TAE	242 g Tris base 57.1 g glacial acetic acid 100 ml of 0.5 M EDTA (pH 8.0) Dissolved in water and the final volume was made upto one litre.
TE Buffer	10 mM Tris/ HCl pH 7,5 1 mM EDTA

3.1.18 Computing facilities used

Hardware

Notebook Intel® Celeron™ CPU	Gericom
Power Macintosh 7600/132	Apple Computers Inc
Laser Jet 4050 N	Hewlett Packard, Palo Alto (USA)
Scanner ScanJet 4C/T	Hewlett Packard, Palo Alto (USA)

Software

Adobe Acrobat 5.0	Adobe System Inc
Adobe Illustrator 10	Adobe System Inc
Adobe Photoshop 6.0	Adobe System Inc
Aida image analyzer	raytest
EndNote 4.0	ISI ResearchSoft

Image gauge	Fuji
Image reader	Fuji
Leica Confocal Software	Leica Microsystems Heidelberg GmbH
Microsoft Office 2000 Professional	Microsoft Corporation
TILLvisION v4.0	TILL Photonics GmbH, Germany
Windows XP	Microsoft Corporation
Image Reader LAS-1000 (Pro Vers. 2.1)	raytest, Straubenhardt

3.2 Methods

3.2.1 METHODS OF CELL BIOLOGY

3.2.1.1 Cell Culture

All cells were grown in a humidified incubator with 5 % CO_2 at 37°C. All media used for cell-culture were pre-warmed to 37°C in a water bath.

For culturing HeLa and Cos cells, Dulbecco's modified essential medium (DMEM) with 10% fetal calf serum (FCS), 2mM L-glutamine and 100U/ml Penicillin/Streptomycin was used.

For cells stably transfected with plasmids encoding Neomycin resistance, the medium was supplemented with 800 µg/ml Neomycin.

3.2.1.2 Passaging of cells by trypsinization

Cells grown to confluence were routinely passaged by trypsinization. The medium from confluent cells was aspirated and the cells were washed once with PBS, followed by incubation with trypsin-EDTA solution on a 37°C metal platform for about 5 min. Detachment of the cells was examined under the microscope. Trypsin was inactivated by adding fresh medium into the flask and the cells were resuspended in this medium. About 1/3 to 1/5 of the volume was left in the flask, while the remaining cells were either used for experimental purposes or discarded. An appropriate

volume of the medium was added to the flask and the cells were placed back into the incubator.

3.2.1.3 Freezing cells for stock maintenance

Freezing medium: Cell culture medium containing 10 % DMSO

The trypsinized cells were collected in a centrifuge tube and were pelleted at 500 g for 5 min. The supernatant was discarded, the cell pellet was resuspended in an appropriate volume of freezing medium. 1ml aliquots of the cell suspension were pipetted into freezing vials and stored at -80°C overnight, before the frozen vials were transferred to liquid nitrogen. Usually from one confluent 35 cm^2 flask, four vials were prepared.

3.2.1.4 Thawing cells from liquid nitrogen

Vials containing frozen cells were taken out of liquid nitrogen, the cap of the vial was loosened in the hood to release the the evaporating N_2, tightened again and thawed in a beaker containing 70 % prewarmed ethanol to 37°C in the waterbath. Subsequently, the cells were pipetted into a centrifuge tube containing chilled cell culture medium, and centrifuged for 5 min at 500 g to remove the DMSO. The cell pellet was resuspended with prewarmed medium, transferred to a new culture flask and kept in the incubator. For transfected cells, on day after thawing, the medium was replaced with medium containing appropriate selection antibiotics.

3.2.1.5 Transfection with Effectene transfection reagent (Quiagen)

Transfection with Effectine reagent was performed according to the manufacturer's prototcol in 4 well plates or in 3 cm dishes. The day before transfection, 7-25 x 10^4 cells were plated onto a 3 cm dish and grown to 10-20% confluency. 1 µg of DNA was diluted in DNA-condensation buffer, Buffer EC, to a total volume of 100 µl. 3.2 µl of Enhancer was added and mixed by vortexing for a second and the resulting solution was incubated at room temperature for 5 min. 10 µl of Effectene transfection reagent was added to the DNA-Enhancer mixture and mixed by vortexing for 10 seconds. The samples were incubated for 10 min at room temperature to allow complex formation. During this incubation time, the old medium was aspirated from the cells and 1.6ml fresh medium was added. 600µl of the medium was added to the

reaction tube containing the transfection complexes and mixed by pipetting up and down. Immediately after mixing, the transfection complex was added dropwise onto the cells and mixed gently by swirling the dish for uniform distribution of the complexes. The cells were incubated at 37°C and 5% CO_2 to allow for gene expression. Next day, the medium was replaced with fresh medium and the cells were used for video-microscopy experiments or for selection of stably transfected clones. 24h following transfection, selection for antibiotic resistant clones was started by adding medium containing an appropriate antibiotic. Antibiotic concentration was gradually increased by 100 µg per day and stopped at 800µg/ml of neomycin. Resistant clones were picked up and cultured as described (3.2.1.1). Stable clones were always maintained in the medium containing resistant antibiotic thereafter.

3.2.2 MOLECULAR BIOLOGY METHODS

3.2.2.1 Cultivation of *E.coli*

Luria Bertani (LB) medium
10 g Bacto-Tryptone
5 g Bacto-yeast extract
5 g NaCl

Dissolved in 900 ml distilled water, pH adjusted to 7.0 with 10 N NaOH, made up the volume to one liter, sterilized by autoclaving and stored at room temperature.

LB-Ampicillin Agar Plates

1.5% of Agar was added to the LB medium and autoclaved. After autoclaving, the medium was let to cool down to 55°C and ampicillin was added to a final concentration 100 µg/ml. This medium was poured into 10 cm petriplates in the hood and left undisturbed for about 30 min to solidify. LB-Agar plates were stored in the cold room.

3.2.2.2 Preparation of competent *E. coli* cells

Buffers

TFB I
30 mM Calcium acetate, pH 5.8
100 mM Rubidium chloride
10 mM Calcium chloride

	50 mM Manganese chloride 15 % Glycerol (w/v)
TFB II	10 mM MOPS, pH 6.5 75 mM Calcium chloride 10 mM Rubidium chloride 15 % (w/v) Glycerol

A 2-3 ml bacterial pre-culture was grown in LB medium at 37°C overnight. 1 ml of the pre-culture was then inoculated into 99 ml of LB medium and grown at 37°C to an OD_{600} of 0.4-0.6. Cells were pelleted at 6,000 RPM for 5 min at 4°C, resuspended in 0.2 culture volume of TFB I and incubated on ice for 5 min. Cells were centrifuged at 5,000 RPM for 5 min at 4°C, the pellet was resuspended in 0.04 culture volume of TFB II and incubated on ice for 30 min. Aliquots of 50 µl were frozen at -80°C.

3.2.2.3 Transformation of *E.coli* competent cells

The competent bacteria were thawed on ice. 100-200 ng of DNA was added to each 50 µl aliquot of competent cells and incubated on ice for 15 min. Cells were subjected to heat shock by incubating at 42°C for 2 min and incubated on ice for 2 min. 1 ml of LB medium was added to the cells followed by incubation at 37°C in the shaker for 40min. Cells were plated on LB-agar plates containing appropriate antibiotic.

3.2.2.4 Preparation of electrocompetent cells

10 % (v/v) Glycerol : 1 ml (1.26 g) of glycerol in 10 ml sterile water

A single *E. coli* colony was inoculated into 10 ml of LB media and allowed to grow overnight under constant shaking at 37°C in an incubator. This pre-culture was inoculated into 1L LB medium and allowed to grow to an OD_{600} of 0.4-0.6 at 37°C. Cells were pre-chilled on ice for 15 min and then pelleted at 5000 RPM for 15 min at 4°C. Pellet was resuspended in 1L of ice cold sterile water and centrifuged as described above. The washing with 500ml water was repeated one more time. The pellet was resuspended in 20ml of 10% glycerol and centrifuged 6000 RPM 15min at 4°C. The bacteria were resuspended in 2ml of 10% glycerol and aliquots of 40, 80 and 160µl were frozen on dry ice and stored at -80°C.

3.2.2.5 Transformation of the electrocompetent cells

SOC medium	0.5 % Yeast extract 2 % Bacto-tryptone 10 mM Sodium chloride 2,5 mM Potassium chloride 10 mM Magnesium sulfate 10 mM Magnesium chloride 20 mM Glucose

For each electroporation 40 µl of the electrocompetent cells were used. 0.5 µg of DNA was added to the cells and the contents were transferred into a pre-chilled electroporation cuvette. The cuvette was placed in the electroporater and pulse was applied. SOC medium was added to the cells and they were allowed to recover in sterile tubes for 30 min at 37°C in a shaker incubator. Cells were plated on LB plates containing appropriate antibiotic.

3.2.2.6 Glycerol stocks of bacterial strains

Bacterial cultures were grown overnight at 37°C in a shaker-incubator. 0.25 ml of sterile 80 % glycerol was taken in 1.5ml tube to which 750 µl of the overnight culture was added. The contents were gently mixed, shock frozen on dry ice and stored at -80° C.

3.2.2.7 Mini preparation of plasmid DNA

Plasmid DNA was isolated from *E.coli* cultures using the kit provided by Qiagen. Isolation was done as follows according to the instructions of the manufacturer.

Buffer P1 (Resuspention buffer)	50 mM Tris/ HCl pH 8,0 10 mM EDTA 100 µg/ml RNase A
Buffer P2 (Lysis buffer)	0.2 M NaOH 1 % SDS
Buffer P3 (Neutralization buffer)	3 M Potassium acetate pH 5.5

A single *E. coli* colony was inoculated into 2 ml of LB medium containing 100 µg/ml of antibiotic and grown overnight at 37°C in a shaker incubator. Cells were pelleted in a

table-top centrifuge at 13,000 RPM for 1 min. Cell pellet was resuspended in 250 µl of buffer P1 and 250 µl of buffer P2 was added, mixed by gently inverting the tube 4-6 times. To this, 350 µl of buffer P3 was added and gently mixed and centrifuged for 10 min at 13,000 RPM in a table-top eppendorf centrifuge. The supernatant was applied onto a QIAprep spin column and centrifuged for 1 min at 13,000 RPM. Flow through was discarded, the column was washed with 0.75 ml of buffer PE and centrifuged again for 1 min. Flow through was discarded and the column was centrifuged for an additional 1 min to remove any residual wash buffer. The column was placed in a clean eppendorf tube and 50 µl of double distilled water was added directly to the centre of the column. The column was let to stand for 1 min and DNA was eluted by centrifuging at 13,000 RPM for 1 min.

3.2.2.8 Midi preparation of plasmid DNA

Buffers P1, P2 and P3 are identical to buffers used for Mini preparations of DNA.

Buffer QBT (Equilibration buffer)	750mM NaCl
	50mM MOPS, pH 7.0
	15% isopropanol (v/v)
	0.15% Triton X-100 (v/v)
Buffer QC (Wash buffer)	1M NaCl
	50mM MOPS, pH 7.0
	15% isopropanol (v/v)
Buffer QF (Elution buffer)	1.25M NaCl
	50mM Tris-HCl pH 8.5
	15% isopropanol (v/v)
Buffer TE	10mM Tris-HCl, pH 8.0
	1mM EDTA

For preparation of up to 200µg of plasmid DNA HiSpeed Plasmid Midi Kit (Qiagen) was used. A single *E. coli* colony was inoculated into 2 ml of LB medium containing 100 µg/ml of antibiotic and grown overnight at 37°C in a shaker incubator. Then the starter culture was inoculated in 150ml medium and grown overnight with vigorous shaking. The bacteria were harvested by centrifugation at 6,000 RPM for 15min at

4°C. The pellet was resuspended in 6ml buffer P1, gently mixed with 6ml buffer P2 by inverting 5 times and incubated for 5min at RT. The lysate was mixed by inverting 5 times with 6ml of buffer P3, poured into the barrel of the QIAfilter cartridge and incubated at RT for 10min. Meanwhile, HiSpeed Midi Tip was equilibrated by applying 4ml buffer QBT. Then the HiSpeed Midi Tip was emptied by gravity flow, the cell lysate was filtered into the Tip and entered the resin by gravity flow. The HiSpeed Midi Tip, containing DNA, was washed with 20ml buffer QC. Afterwards the DNA was eluted with 5ml buffer QF and precipitated by adding 3.5ml isopropanol, mixing and incubating for 5min at RT. Then the eluate/isopropanol mixture was filtered through the QIAprecipitator. The QIAprecipitator was dried by pressing air through the membrane and the DNA was eluted in 0.5ml water.

3.2.2.9 Determining the concentration of DNA

DNA concentration was determined using a spectrophotometer at 260 nm. 1µl DNA was diluted in 100µl water and the absorbance was measured at 260 nm.

Absorbance or optical density (OD) of 1 at 260 nm corresponds to ~50 µg/ml of double stranded DNA or ~40 µg/ml of single stranded DNA and RNA or ~20µg/ml of oligonucleotides. The ratio between the readings at 260 nm and 280 nm (OD_{260}/ OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. Any contamination with proteins or phenol would decrease the values mentioned above.

3.2.2.10 Amplification of DNA by polymerase chain reaction (PCR)

10xPCR buffer	100mM KCl
	100mM Tris-HCl, pH 9.0

The polymerase chain reaction (PCR) with specific primers was used for amplification of plasmid DNA fragments. The reaction mixture contained

Template DNA	100-500ng
10xPCR buffer	10µl
dNTP mixture (1.25mM)	2µl
5'-primer (10pmol/µl)	1µl
3'-primer (10pmol/µl)	1µl
Taq polymerase (U/µl)	0.5µl

Total volume was adjusted to 100µl with ddH_2O.

Usually 25-35 three step cycles were used:

1) 30s at 95°C for template denaturation

2) 90s at 48-58°C annealing of primers to template

3) 1min/kb of the product at 72°C (for *Taq* polymerase) elongation of the PCR product

3.2.2.11 Purification of PCR products

To purify PCR products ranging from 100bp to 10kb from primers, nucleotides, polymerases, and salts, QIAquick PCR purification kit was used. 1 volume of the PCR reaction was mixed with 5 volumes of buffer PB, applied to the QIAquick column and centrifuged 30s at 13,000 RPM in a microcentrifuge. The flow-through was discarded, the column was washed with 750µl buffer PE and centrifuged for one additional time 1min at maximum speed to remove residual washing buffer. DNA was eluted with 50µl ddH_2O.

3.2.2.12 Restriction endonuclease digestion of DNA

The activity of restriction enzymes is measured in terms of `Units' (U).

One unit of restriction enzyme is the amount of enzyme required to completely digest 1 µg substrate DNA in 1h.

Plasmid DNA	0.5 - 1 µg
10 x buffer	1.5 µl
Restriction enzyme	1-2 U
BSA	1 µg/µl (added where essential)

Water was added to a final volume of 15 µl. Reaction mix was incubated at 37°C* for 2 hours.

*Incubation temperatures were set as recommended by the manufacturer (New England Bio Labs Beverly U.S.A.) which varies from enzyme to enzyme. Reaction mix was analyzed on an agarose gel (0.8 to 2 % depending on the size of the DNA of interest)

The buffers and enzymes used were all from New England Biolabs.

The composition of the buffers is as follows:

NEB 1: 10 mM Bis Tris Propane-HCl (pH 7.0), 10 mM $MgCl_2$, 1 mM DTT

NEB 2: 10 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT

NEB 3: 50 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT

NEB 4: 20 mM Tris-acetate (pH 7.9), 10 mM Magnesium acetate, 1 mM DTT,
50 mM Potassium acetate

3.2.2.13 Agarose gel electrophoresis of DNA

The size and purity of DNA was analyzed by agarose gel electrophoresis. Concentration of agarose used for analysis is inversely proportional to the size of the DNA of interest, that is, the larger the DNA the lower the concentration of agarose.

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Agarose concentration (%)	DNA size (kb)
0.7	20 - 1
0.9	7 – 0.5
1.2	6 – 0.4
1.5	4 – 0.2
2.0	3 – 0.1

Gel loading buffer (10x)

0.25 % (w/v) Bromophenol blue

40 % Saccharose in 1x TAE

Agarose was weighed and dissolved in 1 x TAE by boiling in a microwave oven. The agarose solution was allowed to cool down to 60°C or lower before ethidium bromide was added to a final concentration of 0.5µg/ml. The solution was poured into the agarose gel cassette and allowed to polymerize completely. The sample DNA was mixed with gel loading buffer and loaded into the lane. The gel electrophoresis was carried out at 100 V. Ethidium bromide is a fluorescent dye which contains a planar group that intercalates between the stacked bases of the DNA. The fixed position of

this group and its close proximity to the bases cause dye bound to DNA to display an increased fluorescent yield compared to that of the dye in free solution. Ultraviolet radiation at 254 nm is absorbed by the DNA and transmitted to the dye; radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red orange region of the visible spectrum. Hence DNA can be visualized under a UV transilluminator. The gel was photographed using a gel documentation system.

3.2.2.14 Extraction of DNA fragments from agarose gels

DNA fragments separated by agarose gel electrophoresis were extracted and purified by means of QIAquick gel extraction kit. DNA fragment of interest was excised from the agarose gel. Resulting gel slice was weighted, submerged into 3 volumes of buffer QG to 1 volume of gel and incubated at 50°C for 10min (or until the gel slice has completely dissolved). The DNA solution was applied to the QIAquick column and centrifuged for 1min. The flow-through was discarded, the DNA bound to the column was processed further as described in 3.2.2.11.

3.2.2.15 Ligation of DNA fragments

1x T4 DNA ligase reaction buffer	50mM Tris-HCl, pH 7.5
	10mM MgCl2
	10mM dithiothreitol
	1mM ATP
	25µg/ml BSA

The activity of ligases is measured in terms of `Units' (U). One NEB unit is defined as the amount of enzyme required to give 50% ligation of Hind III fragments of λ DNA (5'-termini concentration of 0.12µM) in 20µl of T4 DNA ligase reaction buffer in 30 min at 16°C.

T4 DNA ligase (400 U/µl)	1.5µl
Vector DNA fragment	10-15ng
Insert DNA fragment	100-200ng
10 x buffer	2 µl

Water was added to a final volume of 20 µl. Ligation mixture was incubated at RT for 2 hours or at 16°C overnight.

3.2.2.16 Cloning of full length and truncated TIP47 cDNA

Full length *Homo sapiens* TIP47 cDNA was cloned by RT-PCR using total RNA isolated by the RNAeasy method (Quiagen) from HeLa cells and primers matching to the coding region of human TIP47 mRNA. 5'-primers included XhoI cloning site and HA-, RGS-His- or no tag; 3'-primers introduced HA-, RGS-His- or no tag as well as BamHI restriction site after the stop codon (Table 3.2). These primers were necessary to generate proteins, containing the tag at the C- or N-terminus:

HA-TIP47	TIP47-HA
His-TIP47	TIP47-His

Amplified DNAs were cloned into the pMPSV-EH vector with XhoI/BamHI cloning sites and sequenced (3.2.2.18).

TIP47 protein was conventionally divided into 3 parts (ссылка на резельтаты):

The PAT domain	1-116 aa residues
homologous to LD targeting domain	117-239 aa
of ADRP (McManaman et al., 2003)	
coiled-coil rich region	240-433 aa

So 5 constructs for mammalian expression of truncated proteins

HA-TIP47 1-116

HA-TIP47 1-239

HA-TIP47 117-239

TIP47-HA 117-434

TIP47-HA 240-434

were generated as described above, sequenced (3.2.2.18) and expressed in HeLa cells (3.2.1.5).

Table 3.2. Primers used for cloning of full length and truncated TIP47. XhoI and BamHI cloning sites (5'- and 3'-, respectively) are underlined, start and stop codons are shown in lowercase letters. HA- and His-tags are italicized.

Primer specificity	Orientation	Sequence 5'→3'
Including XhoI site and start codon	5'	TCTGCCCGCTCGAGCatgTCTGCCGACGGGG
Including BamHI site and stop codon	3'	TCCGCGGATCCCctaCTTCTTCTCCTC
Introducing RGS-His-tag at N-terminus of full length TIP47	5'	TCTGCCCGCTCGAGCatg*AGAGGATCGCATCACCATCACCATCAC*ATGTCTGCCGACGGGGC
Introducing HA-tag at N-terminus of full length TIP47	5'	TCTGCCCGCTCGAGCatg*TATCCGTATGATGTGCCTGACTACGCA*atgTCTGCCGACGGGGC
Introducing RGS-His-tag at C-terminus of full length TIP47	3'	TCCGCGGATCCCcta*GTGATGGTGATGGTGATGCGATCCTCT*CTTCTTCTCCTCCGGGG
Introducing HA-tag at C-terminus of full length TIP47	3'	TCCGCGGATCCCcta*TGCGTAGTCAGGCACATCATACGGATA*CTTCTTCTCCTCCGGGG
Introducing stop codon after K116	3'	TCCGCGGATCCCctaCTTCTCCGTGGGCTGCTG
Introducing start codon and HA-tag before V117	5'	TCTGCCCGCTCGAGCatg*TATCCGTATGATGTGCCTGACTACGCA*GTCCTGGCGGACACCAAG
Introducing start codon before V117	5'	TCTGCCCGCTCGAGCatgGTCCTGGCGGACACCAAG
Introducing stop codon after L239	3'	TCCGCGGATCCCctaCAGACGTACGAAGTAGCTC
Introducing start	5'	TCTGCCCGCTCGAGCatgGGCTCCCTGTC

codon before G240		GGAGAG

3.2.2.17 Cloning of TIP47 RNAi constructs and establishing of RNAi HeLa cell line

GeneSilencer, a plasmid-based system for RNA interference (RNAi), was used to decrease TIP47 expression level in HeLa cells. The technique is based on ability of small interfering RNAs (siRNAs) to suppress gene expression. Introduction of double-stranded RNA (dsRNA), that are homologous in sequence to a gene, has proven to suppress gene expression without producing a non-specific cytotoxic response (Elbashir et al., 2001). Inserts cloned into GeneSilencer vector pSHH express RNAs under U6 promoter in the transfected mammalian cells. The RNAs are expressed as fold-back stem loop structure, which are processed into the siRNAs. The oligonucleotide inserts were designed as follows: target region of TIP47 mRNA in direct and reversed orientation were separated by short loop sequence (Brummelkamp et al., 2002). 4 nucleotide overhang for ligation with SalI/XhoI site and TTTTT as terminator of transcription were added at the 5'- or 3'- end of the resulting sequence, respectively. Overhang for ligation with XbaI site was added to the complementary sequence at 5'-end before AAAAA. The oligonucleotides (Table 3.3) were annealed 10min at 95°C and ligated with linearized vector pSHH. These plasmids were sequenced (see chapter 3.2.2.18) and transfected in HeLa cell line. Clones resistant for upto 800 µg/ml neomycin were picked and cultured for further experiments. Decrease in TIP47 expression level was tested by western-blot using commercial guinea pig or affinity purified rabbit anti-TIP47 antibodies (kind gift of Dr. S.R.Pfeffer).

Table 3.3. Olygonucleotides used for plasmid based RNAi. Underlined letters represent sticky ends for XhoI/SalI sites (5'-primer) and XbaI sites (3'- primer). Capital letters show target region of the gene in direct and reverse orientation. The stem loop is shown in lowercase letters.

Oligonucleotide specificity	Orientation	Sequence 5'→3'
RNAi of TIP47 1	5'	<u>TCGA</u>GGACACGGTGGCCACCCAAttcaaga gaTTGGGTGGCCACCGTGTCCTTTTT

RNAi of TIP47 1	3’	CTAGAAAAAGGACACGGTGGCCACCCAAt ctcttgaaTTGGGTGGCCACCGTGTCC
RNAi of TIP47 2	5’	TCGACCCGGGGCTCATTTCAAACttcaagag aGTTTGAAATGAGCCCCGGGTTTTT
RNAi of TIP47 2	3’	CTAGAAAAACCCGGGGCTCATTTCAAACt ctcttgaaGTTTGAAATGAGCCCCGGG

3.2.2.18 DNA sequencing

The sequencing of DNA was based on the method of Sanger and coworkers (1977) with some modifications. ABI PRISM® Rhodamine Terminator Cycle sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Weiterstadt, Germany) was used for sequencing. The kit contains rhodamine derivatives of di-desoxynucleotides which differ in their absorption and emission spectra depending on the ddNTP. The rhodamine-labeled ddNTPs were incorporated into the synthesized DNA during elongation of DNA chains in a sequence PCR. The reaction mixture contained:

Terminator Ready Reaction Mix 2µl
Plasmid DNA 200-300ng
Primer 1pM

ddH_2O was added to a final volume of 10 µl.

The primers used for the reaction are gene-specific or correspond to sequences present in vectors themselves (Table 3.4.).

Amplification cycle, as follows, was repeated 25 times.

96°C 10s
50°C 5s
60°C 4min

After the PCR, the DNA was precipitated with ethanol to remove free rhodamine-labeled ddNTPs. 2µl of sodium acetate pH 5.2 were mixed with 50µl 96% ethanol. The PCR product was added to the solution, mixed, incubated for 10min at room temperature and centrifuged 13,000 RPM for 20min. The pellets were briefly washed with 250µl of 70% ethanol and spin down for 10min at 13,000RPM. The pellets were dried for 5min and resolved in 25µl of ddH_2O. The samples were kept at -20°C until

sequencing in ABI PRISM^{TM} 310 or 3100 Genetic Analyser (Perkin Elmer Life Sciences Ltd, Cambrige, UK).

Table 3.4. Primers used for sequencing.

Primer specificity	Orientation	Sequence 5'→3'
pBEH and pMPSV	5'	GTTAACTGGTAAGTTTAG
pBEH and pMPSV	3'	GCATTCTAGTTGTGGTT
pMPSV-EH before XhoI cloning site	5'	CACAACCCCTCACTC
TIP47	5'	GAGAAGGGAGTGAGG
TIP47	5'	TGTCGGAGGCGGTG
TIP47	5'	GAACAGAGCTACTTCG
TIP47	5'	GGAACCAGAAGCAGC
pSHH	5'	GTTTTAAAATTATGTTTTAAAATGG
pSHH	3'	GGTAACCGACGTCAAGC

3.2.3 BIOCHEMICAL METHODS

3.2.3.1 Analysis of proteins

3.2.3.1.1 Protein determination

Bovine Serum Albumin (BSA) stock solution 1 mg/ml

A standard curve was made using BSA in the concentration range of 2 -16 µg. 3-10 µl of the sample was used for the protein estimation. When the sample contained no detergents, the volume was made up to 800 µl with water. 200 µl of the BIORAD reagent was added and the samples were incubated for 3 - 5 min at room temperature, 200 µl from each tube was pipetted into ELISA strips and optical density was measured at 595 nm in the ELISA reader.

3.2.3.1.2 Solubilisation of proteins

2 x Laemmli buffer 125 mM Tris-Cl, pH 6.8

4 % SDS

0.00 4% Bromophenol blue

20 % (w/v) Glycerol

20 mM DTT

Proteins were resuspended in 1x Laemmli buffer. The resuspended proteins were boiled at 95°C for 5 min, cooled on ice for 1 min, centrifuged at 13,000 RPM for 2 min and resolved by SDS-PAGE.

3.2.3.1.3 TCA precipitation of proteins

Trichloroacetic acid (TCA) solution

(100 %)	100 g of TCA crystals was weighed and the volume was made up to 100 ml with water
2 % Sodium acetate in ethanol	2 g of Sodium was dissolved in small amount of water and volume was made up to 100 ml with ethanol

The proteins were precipitated by adding TCA to a final concentration of 10% and

incubated on ice in the cold room overnight. The precipitated samples were centrifuged at 13,000 RPM, 10 min, 4°C. The supernatant was discarded and the pellets were washed 500 µl of 2 % sodium acetate solution in ethanol. The samples were again centrifuged as above, supernatant was discarded and the pellets were air-dried for 10-15 min at RT. The pellets were resuspended in 1x SDS-PAGE buffer, boiled and loaded on SDS-PAGE for further analysis.

3.2.3.1.4 SDS Poly-Acrylamide Gel Electrophoresis (SDS-PAGE)

Buffers

Running buffer (1x)	25 mM Tris
	192 mM Glycine
	0.1% SDS
	Volume was made up with ddH_2O.
Separating gel buffer (4x)	1.5 M Tris-Cl pH 8.8
	0.4 % SDS
	0.4 % APS
	Volume was made up with ddH_2O.
Stacking gel buffer (4x)	0.5 M Tris-Cl pH 6.8
	0.4 % (w/v) SDS
Acrylamide Solution	30 % (w/v) Acrylamide
	0.8 % (w/v) Bisacrylamide
Ammonium per sulphate	10 % (w/v)

Recrystallised ammonium peroxidisulphate (APS) was dissolved in double distilled water. This was prepared fresh every week.

Preparation of acrylamide solution for SDS-PAGE (for one minigel - 8ml).

All volumes are in ml unless indicated otherwise.

Gel (%)	5	10	12.5	15	3 ml Stacking Gel (4.5 %)
Acrylamide/ Bis-acrylamide	1.33	2.67	3.3	4	0.45
4 x resolving gel buffer	2	2	2	2	4x stacking gel buffer 0.45
Water	4.58	3.2	2.6	1.9	1.77
Temed	8 µl	8 µl	8 µl	8 µl	4 µl
10% APS	80 µl	80 µl	80 µl	80 µl	40 µl

SDS-PAGE is a biochemical technique wherein proteins are separated based on their molecular weight. The gel is discontinuous in that it has a stacking gel which is present on top of the resolving gel. SDS-PAGE can be used to separate proteins based on their native size (native SDS-PAGE) or their sub unit size (reducing SDS-PAGE). Two clean glass plates were fixed together with a spacer of appropriate thickness (0.75-1.5 mM) using holders or clips. The resolving gel was poured and isobutanol was layered on top of it. The gel was allowed to polymerise for about 10 min and isobutanol was thoroughly washed off using double distilled water. The stacking gel was poured on top of the resolving gel and a comb, of appropriate size and appropriate number of wells, was placed in the stacking gel and left undisturbed for about 10 min. After polymerisation of the stacking gel, the combs were removed and the wells were cleaned with double distilled water to wash off any unpolymerised acrylamide. Boiled and denatured protein samples were loaded into the wells, the gel was placed in a tank with electrophoresis buffer and run at 100 -125V for 2 h.

3.2.3.1.5 Coomassie blue staining of Polyacrylamide gels

Coomassie blue stain 0.5 % (w/v) Coomassie blue
50 % (v/v) Methanol
10 % (v/v) Acetic acid
in double distilled water

Destaining solution	50 % Methanol and 10 % glacial acetic acid dissolved in double distilled water.

The gel was stained in Coomassie blue solution at room temperature for 1-2 hours and destained using the destaining solution overnight. After complete destaining the gel was washed with double distilled water for 15 min and then dried in a gel drier.

3.2.3.1.6 Silver staining of polyacrylamide gels modified for MS

Buffers

Fixer	50% MeOH
	12% acetic acid
	0.05% formalin
	in ddH_2O
Washing solution	35% EthOH in ddH_2O
Sensitizing	0.02% $Na_2S_2O_3$ in ddH_2O
Silver nitrate	0.2% $AgNO_3$
	0.076% formalin in ddH_2O
Developer	6% Na_2CO_3
	0.05% formalin
	0.0004% $Na_2S_2O_3$ in ddH_2O
Stop solution	50% MeOH
	12% acetic acid in ddH_2O

The polyacrylamide gel was fixed for 2h in fixer and washed 3 times with 35% EthOH for 20min each. Then the gel was sensitized for 2min 0.02% $Na_2S_2O_3$ and washed with ddH_2O 3 times for 5min. In order to stain the gel was submerged into silver nitrate for 20min, washed twice with ddH_2O for 1min and developed in sodium carbonate solution. Staining was stopped by 5min incubation in the stop solution. The gel was stored in 10% EthOH at 4°C till mass spectrometry analysis.

3.2.3.1.7 Western blot analysis (wet method)

Blot buffer	25 mM Tris
	192 mM Glycine
	20 % Methanol
	were dissolved in water.
PBST	PBS containing 0.05% Tween-20
Blocking buffer	PBST containing 5% milk powder

Primary antibodies	**Final concentration**
anti-gamma adaptin	1:5,000
anti-alpha adaptin	1:5,000
anti-MPR46	1:1,000
anti-TIP47	1:1,000

Secondary antibody	**Final concentration**
HRP (Horseradish peroxidase) conjugated Goat anti-guinea pig, anti-rabbit and anti-mouse antibody	1:5,000

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membrane for western blot analysis as follows. 2 sheets of whatman paper and one piece on nitrocellulose membrane were cut according to the size of the gel. A pad and one sheet of paper was immersed in the blot buffer and placed in the chamber. The gel was also soaked in the blot buffer before placing on the paper. Then the membrane dipped in the blot buffer was placed on top of it and the air bubbles were removed. Another whatman paper sheets was dipped in the buffer and placed on top of the gel. A glass pipette was rolled on the set up to remove air bubbles and the second pad was placed on top of the stack. The proteins were transferred onto the membrane using current at 150V for 90 min.

After the transfer, membrane was briefly washed with PBS, incubated in PBST containing 5% milk powder for 1 hour at room temperature or o/n at 4°C to block all non-specific interaction sites on the membrane. After blocking, the primary antibodies suitably diluted in PBST, were added onto the membrane and incubated 1-4h RT or overnight in the cold room on a rocker. The non-specifically bound antibody was washed off by incubating the blot on a rocker with PBST, changing the buffer once every 10 min for three times. The membrane was then incubated with the secondary antibody for 1 hour at room temperature and the membrane was washed 3 times, 10 min each, with PBST. The proteins were detected by chemiluminescence kit after an additional brief washing of the membrane with PBS.

3.2.3.1.8 Detection technique. Chemiluminescent Substrate (PIERCE)

The nitrocellulose membrane was incubated at room temperature for 1 min in enhancer and peroxide solutions mixed in a 1:1 ratio. The membrane was wrapped in a polythene sheet and signals were detected using a CCD camera.

3.2.3.1.9 Stripping the antibody from the membrane

Stripping buffer	100mM β-mercaptoethanol 2% SDS 62.5mM Tris-HCl pH 6.7

The membrane was washed once with PBS for 5 min to wash off the chemiluminescent substrate, submerged in stripping buffer and incubated at 50-70°C for 30min with occasional agitation. Then the membrane was washed twice with PBST for 10min using large volume of the buffer, incubated in blocking buffer for 1h followed by antibody of interest for next round of chemiluminescence.

3.2.3.2 Metabolic labeling of cells with [^{35}S]-Methionine/Cysteine

Solutions

dFCS	heat inactivated dialyzed fetal calf serum
Starvation medium	DMEM without Methionine /Cysteine supplemented with 5 % dFCS and 2 mM Glutamine
^{35}S-Methionine/Cysteine	10 µCi/µl

Labelling of starved cells with radioactive methionine and cysteine leads to the incorporation of these amino acids into newly synthesized proteins and helps in the detection of the protein of our interest. Cells were grown in normal DMEM medium till 70 - 80% confluency. Before labeling, the cells were washed with PBS 3 times and incubated with starvation medium for one hour. The cells were again washed once with PBS and incubated with labeling medium (starvation medium containing normally 100 µCi/ml of ^{35}S-Methionine/Cysteine) for required time periods. In pulse-chase experiments, after pulse period, the cells were chased after washing twice with PBS and addition to the dishes normal culture medium DMEM with 10%FCS. The labeled cells were processed as per the protocol for the desired experiments.

3.2.3.3 Phosphorylation of TIP47 in vivo

Solutions

dFCS	heat inactivated dialyzed fetal calf serum
Starvation medium	DMEM without phosphate and piruvate, suplemented with 5 % dFCS and 2 mM Glutamine
[γ-^{32}P]-ATP	10mCi/ml

To analyse the putative dependence of TIP47 phosphorylation level on nutritional status of the cell, HeLa cells were plated onto three 3 cm dishes and grown overnight. Then two of the dishes were continuously incubated under standard cell culture conditions, later washed five times with PBS and incubated for 1h at 37°C in the starvation medium (samples 1, 2) or, alternatively, preincubated in the same media containing 600µM oleic acid complexed to BSA (6:1) (sample 3). After the inculation starvation media were aspirated, cells were supplied with 600µl of the labeling medium containing 500µCi of [γ-^{32}P]-ATP (sample 1, 3) or with the same labeling medium supplemented with 600µM oleic acid (sample 2) and incubated for 6h. After the labeling cells were processed for immunoprecipitation as described below (3.2.3.4).

3.2.3.4 Immunoprecipitation of TIP47

Solutions	Working concentration
Homo Buffer	50mM Tris-HCl, pH 7.4
	1% Triton X-100
	0.5% Sodium Deoxycholate
	300mM Sodium chloride
	with protease inhibitor cocktail
0.1x PBS	1:10 diluted PBS
PANSORBIN	Heat killed and inactivated *Staphylococcus aureus* cell suspension in PBS
PANSORBIN	*Staphylococcus aureus* cells
Protein-A sepharose	Washed five times with PBS
NaCl - Tween 20 solution	1M NaCl
	0.5% Tween 20

“Cold” HeLa cells grown on 6cm dishes or labeled as described previously () were washed three times with PBS, scraped and pelletted by low speed centrifugation. The cells were resuspended in 1ml of Homo buffer and incubated for 10min at 4°C rotating. The resulting cell lysates were centrifuged for 15min with maximal speed in a table-top centrifuge. The supernatant was transferred into new tubes and mixed with 2µl of rabbit preimmune serum and 40 µl of washed Pansorbin and incubated on a spinning wheel in cold room for 1 hour. After this pre-clearing, samples were centrifuged for 30 min at 13,000 RPM, 4°C. The supernatant was carefully transferred to new tubes, 3 µl of rabbit anti-TIP47 antibody were added to each tube and incubated on spinning wheel in cold room overnight for the formation of specific antigen-antibody complexes. 40 µl of washed Protein-A agarose (50% solution in PBS) were added and the incubation was continued for another one hour. The samples were centrifuged for 2 min at 13,000 RPM, 4°C. Supernatants were collected in fresh tubes and were frozen at -20°C. The pellets were washed five times with NaCl-Tween20 solution and once with 1/10 PBS. 20µl of LBS were added to each sample, boiled for 10min, centrifuged for 2 min at 13000 RPM. The solubilized proteins were separated by a 10% PAGE.

3.2.3.5 Immunoprecipitation of MPR46

Solutions	Working concentration
TBS	10 mM Tris-Cl, pH 7.4
	150 mM NaCl
Buffer A	0.1 M NaAc
	0.2 M NaCl
TIN buffer	0.5% TX100
	50mM Imidazole pH 7.0
	150mM NaCl & protease inhibitors
Immunomix (IMM)	1% Triton X-100
	0.5% sodium deoxycholate
	10 mM Sodium phosphate buffer, pH 7.4
	0.15 M Sodium chloride
Precipitation immunomix	10% BSA and 0.2% SDS in Immunomix with
(PIM)	protease inhibitors
Neufeld buffer	10 mM Tris-Cl, pH 8.5
	0.6 M Sodium chloride
	0.1% SDS
	0.05% NP- 40
2M KCl-IMM	2 M KCl in IMM

Cells were metabolically labeled overnight as described earlier (3.2.3.2). After labeling, cells were washed twice with cold PBS, scraped in 1 ml PBS (500 µl x 2) and centrifuged for 5 min at 3,000 RPM, 4°C. The supernatant was discarded, the pellet was resuspended in 400 µl of TIN buffer, sonicated 3 x 10s and incubated on vibrax in cold room for 20 min to solubilise all membrane proteins. After solublilisation, the samples were centrifuged at 13,000 RPM, 20 min, 4°C. The supernatant was transferred to a new tube and the insoluble pellet was discarded. To the supernatants, 0.8 volume of PIM, 3 µl of rabbit pre-immune serum and 50 µl of unwashed Pansorbin were added and incubated on a spinning wheel in cold room for 2 hours. After this pre-clearing of non-specifically interacting proteins, the samples

were centrifuged at 13,000 RPM, 10 min, 4°C. Supernatant was carefully transferred to a new tube. 3 µl of rabbit anti-MPR46 antibody was added to the samples and incubated in the cold room on the spinning wheel overnight. 40 µl of washed Protein-A sepharose beads were added and the incubation was continued for two hours in the cold room on a spinning wheel. The antigen-antibody complexes bound to the beads were pelleted at 6,000 RPM, 5 min, 4°C. Supernatant was discarded and the beads were washed with Neufeldbuffer, IMM, IMM containing 2 M KCl, and 1/10 PBS. Washes were performed at 5,000 RPM, 4°C for 5 min. After last wash, the supernatant was completely removed and 60 µl of 1x Laemmli buffer with DTT was added and boiled for 5 min at 95°C, incubated on ice for few seconds and centrifuged for 2 at 13,000 RPM. The proteins were resolved on a 10 % SDS-PAGE (Minigels) at 100-125 V for about 2 hours (3.2.3.1.3). The gel was fixed in destaining solution for 20 min, washed with water twice, 5 min each, dried in a BioRad gel drier and exposed onto a phosphoimager screen. The bands were visualized by autoradiography.

3.2.3.6 Isolation of lipid droplets by subcellular fractionation

Lysis buffer
10 mM Tris-HCl pH 7.4
1 mM EDTA
containing protease inhibitors:
40 µg/ml Aprotinin
10 µg/ml Leupeptin
1 µM Pepstatin-A

Hela or TIP47 RNAi cells were plated onto 15 cm dishes ($45x10^4$ cells per dish) and grown 24h in normal medium. Then the medium was aspirated, cells were supplemented with fresh medium with or without 600µM oleic acid and incubated for 12h. Cells were washed twice with cold PBS, scraped in PBS and pelleted by low speed centrifugation. Then they were disrupted by hyperosmotic shock according to an established procedure (Wolins et al., 2001). The cell pellets were dispersed by vortexing, concurrent with the dropwise addition of 2M (70% (w/w)) sucrose dissolved in lysis buffer at room temperature. The cells were then incubated on ice for 10min, during which time they were vortexed for 30 s every 2 min. To maximize the osmotic

shock, 3ml of lysis buffer were added rapidly while vortexing the sample. The homogenate was incubated for an additional 10min on ice and vortexed for 30s every 2min; the cells were then further disrupted by passing them through a 27-gauge needle four times.

Then lipid droplets (LD) were isolated essentially as described by Yu and coworkers (2000). The cell homogenate was centrifuged for 10min at 1500g at 4°C to pellet nuclei and intact cells. The supernatant was transferred to a 12-ml ultracentrifugation tube and overlaid sequentially with 2ml each of 0.27 M sucrose in the lysis buffer, 0.135M sucrose in lysis buffer and lysis buffer itself as a top solution. Following centrifugation at 154 324g for 1h, 7 fractions of 1.5ml were collected from top to bottom: the buoyant LD (Nos. 1 and 2), the mid zone (No. 3) between LD and cytosol, and the cytosol (Nos. 4-7).

For analysis of the LD phospholipids and proteins, fraction No 1, containing most of the LD, was transferred into 50ml tube, mixed with 6ml methanol by vortexing and spin down 5000 RPM 1min. Then 1.5ml chloroform were added to the solution, mixed by vortexing and spin down 5000 RPM 1min. The last, 4.5ml ddH_2O were added to the mixture, vortexed for 5 s and centrifuged for 15min at 5000 RPM in order to separate phases. The upper phase was discarded, the lowest phase, containing extracted lipids, was directly subjected to mass spectrometry analysis, the proteins from the phase border were transferred into a new 1.5ml tube, dried in the air, resolved in 50µl LSB, boiled for 10min and loaded on a SDS-PAGE (3.2.3.1.3).

3.3 Microscopy

3.3.1 Indirect Immunofluorescence

3.3.1.1.1 Fixing cells by Methanol

Solutions

PBS	10 mM Na_2HPO_4, pH7.4, 150 mM NaCl
10% goat serum	10% goat serum in PBS
Methanol	Cooled by storing at -20°C
DAKO	Mounting medium stored at 4°C

The cells were plated on cover slips in a 24 well plate and grown till 50-70% confluency. Then they were washed twice with PBS. Solutions from the wells were sucked out using a tube connected to a vacuum pump and the following solutions were immediately added onto the cells. They were never let to dry up. The cells were fixed with methanol from -20°C for 5 minutes. After fixation cells were washed 3 times with PBS at room temperature and incubated for 20min at 4°C. During this incubation, the primary antibody was diluted 1:30-1:3000 in PBS. About 30µl of the antibody solution was added on to each cover slip and incubated for 1h at 37°C. Care was taken not to disturb the plate during incubation with the antibodies. After incubation with the primary antibody cells were again washed 3 times with PBS and incubated 20min with 10% goat serum to block non-specific binding. The cover slips were washed again twice with PBS and incubated for 1h at 37°C with 30µl of 1:50-1:200 diluted (in PBS) secondary antibody conjugated with a fluorescent dye (Alexa 633, Cy2, Cy3 or others depending on the experiment design). After this incubation, cells were washed two times with PBS and once with double distilled water. Meanwhile, clean glass slides were labeled and a drop of DAKO solution was added and kept aside. The cover slip was carefully taken out of the wells using syringe and a forceps, excess water was absorbed by gently touching it to a tissue paper and was placed on DAKO solution on the glass slide with the cell side facing the solution. These glass slides were incubated overnight at room temperature in dark. Next day, the border of the cover-slip was sealed with a nail-polish and after few minutes the

cells were observed under fluorescence microscope. Slides were stored at 4°C for further analysis.

3.3.1.1.2 Fixing cells by paraformaldehyde (PFA)

Solutions

PBS	10 mM Na_2HPO_4, pH 7.4, 150 mM NaCl
3%PFA	3 % paraformaldehyde in PBS The desired amount of PFA was weighed and a small amount of PBS was added, heated to 60°C on a heating block with constant mixing and after 10 min, 1-2 drops of 1 N NaOH was added, solution gets cleared immediately and the volume was made up to the desired amount with PBS and the pH was adjusted to 7.4 with HCl.
PBS/Saponin	0.5% saponin in PBS

DAKO Mounting medium stored at 4°C

Cells were grown on sterile cover slips in a 24 well plate to 50-70% confluency, washed twice with PBS and fixed with 3% PFA for 20 min at room temperature. The fixed cells were again washed twice with PBS. Cells were permeabilized with 0.5% Saponin in PBS for 3 x 10 min. Afterwards cells were washed twice with PBS and incubated with primary and secondary antibodies as well as with 10% goat serum as described above (3.3.1.1.1).

3.3.1.2 Staining of LD with Nile red

PFA fixation was found the best to preserve LD (DiDonato and Brasaemle, 2003) so it was used for further experiments. Nile red was reported as a selective fluorescent stain for the detection of intracellular lipid droplets by fluorescent microscopy (Greenspan et al., 1985a). Nile red was added to the secondary antibodies solution in dilution 1:1000.

3.3.1.3 Transferrin endocytosis

Transferrin is endocytosed from the medium by the transferrin receptor at the plasma membrane. The internalized transferrin is then delivered to the intracellular

compartments and the transferrin receptor recycles back to the plasma membrane for further rounds of sorting. The kinetics of transferrin endocytosis and recycling has been well characterized and hence they are widely used as markers of the early and recycling endosomes.

HeLa and Cos cells were grown on coverslips to 50-60% confluency under standard cell culture coditions. The medium was replaced by a serum free medium containing 0.5% BSA (ultra pure) and incubated for further one hour. 0.1 µM of Alexa633 conjugated mouse transferrin was added to the wells and mixed with a pipette for uniform concentration of transferrin in the medium and continued incubation at 37°C for 1h. This is the time required for the endocytosed transferrin to reach late endosomes. The cells were immediately shifted onto the ice and processed for immunoflorescence by PFA fixation as described in (3.3.1.1.2). The coverslips were incubated with polyclonal anti-TIP47 primary antibodies and FITC-conjugated anti-rabbit secondary antibodies.

4 Results

4.1 TIP47 localization

4.1.1 TIP47 detection in HeLa cells

There were two reasons to thoroughly examine a number of proteins recognized by anti-TIP47 antibodies used in our experiments. On one hand, several proteins differing in molecular weight are recognized by an antiserum against pp17/TIP47, perhaps as a result of alternative splicing, as previously reported (Than et al., 2003; Than et al., 1998; Than et al., 1999). On the other hand, specificity of anti-TIP47 antibodies and localization of the protein in cells were a mater of a recent debate (Barbero et al., 2001; Wolins et al., 2001).

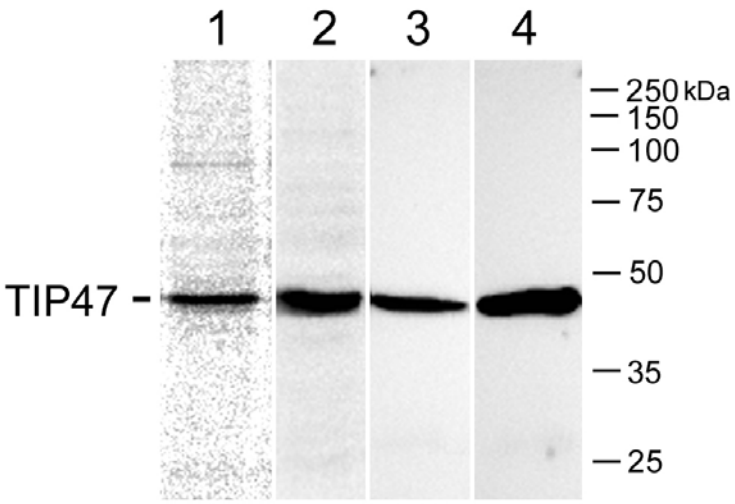

Figure 4.1. Specificity of TIP47 antibodies. Two antibodies from different sources were used in our experiments to detect TIP47 in whole HeLa cell lysates or immunoprecipitation. In lane 1 a single protein is visible, which was immunoprecipitated from metabolically labeled HeLa cells and has a molecular weight corresponding to the MW of TIP47. The same antibody was used to detect TIP47 via western-blotting in total cell lysates (lane 2). A band of exactly the same size was detected by another, commercially available antibody (lane 3), indicating that both Abs detect the same protein. This was further confirmed by using antibody 2 to detect the protein precipitated by antibody 1. As shown in lane 4, antibody 2 clearly recognizes the protein TIP47, which was first immunoprecipitated by antibody 1, revealing that both antibodies are against the same protein.

In our experiments we used two anti-TIP47 antibodies: commercially available polyclonal antibodies (guinea pig) raised against a synthetic polypeptide corresponding to the N-terminal residues 1-16 of TIP47, and secondly, an affinity

purified rabbit serum donated by S.R. Pfeffer (Barbero et al., 2001). Both sera were checked for their specificity on total HeLa cell lysates. As it is shown in Figure 4.1 both antibodies recognize the same protein (and only one) in western blotting as well as after immunoprecipitation. Thus, both antibodies could be used for further analysis of TIP47 in HeLa cells.

4.1.2 Localisation of endogenous TIP47

The distribution of endogenous TIP47 was first analyzed by immunofluorescence. Under standard cells culture conditions, TIP47 positive structures were not homogenously distributed among HeLa cells (Figure 4.2.). While some cells possess plenty of TIP47-positive structures, others exhibit moderate amount or no TIP47-positive structures at all.

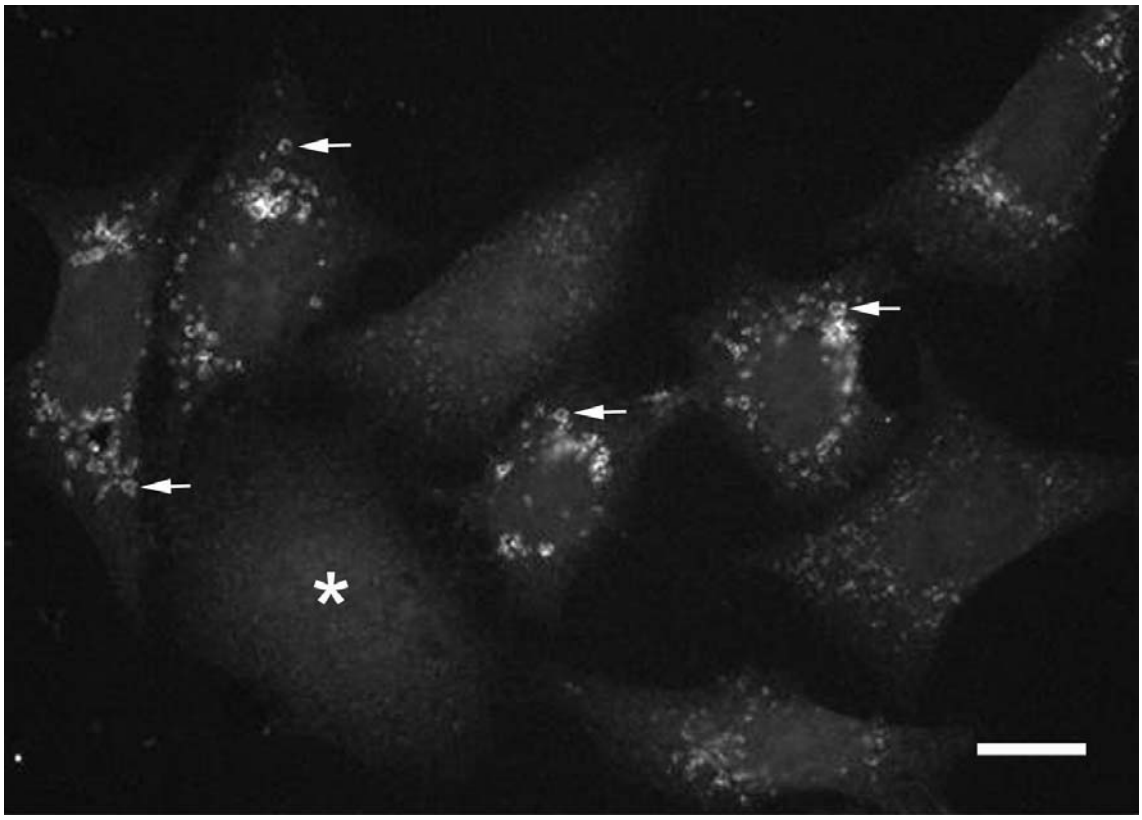

Figure 4.2. TIP47 distribution in HeLa cells. HeLa cells grown under standard cell culture conditions were fixed and further processed for immunofluorescence staining of TIP47. It was interesting to note that the distribution of TIP47 was not homogenous among all cells. While some cells exhibited a bright staining of TIP47, often found in “ring-like” structures (see arrows), other cells exhibited only a low degree of labeling and also some cells did not show any staining at all (see asterisk). Bar: 20µm.

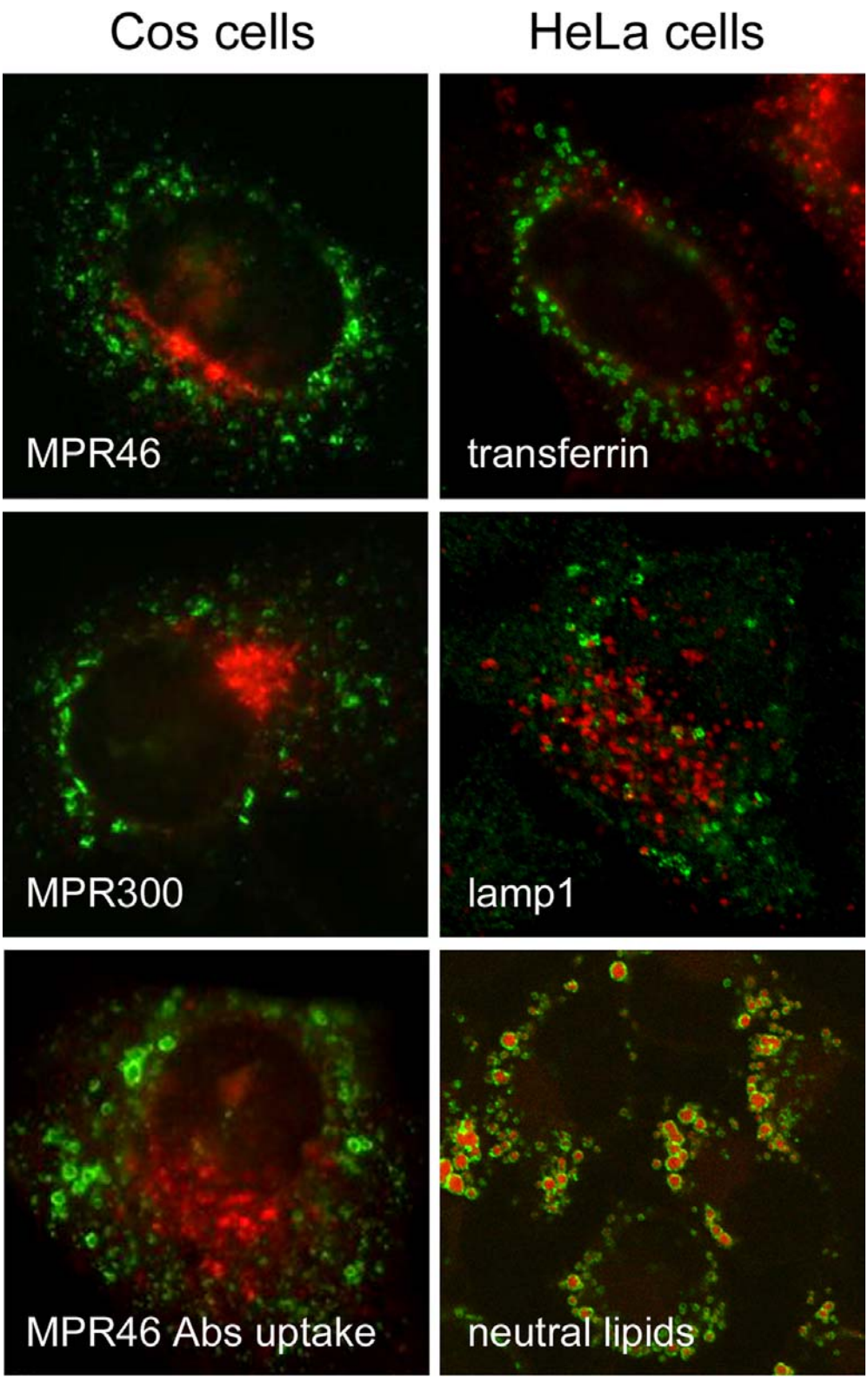

Figure 4.3. TIP47 does not colocalise with markers of the TGN, endosomes and lysosomes, but decorates the surface of lipid droplets in Cos and HeLa cells. Endogenous TIP47 is shown in green; MPR46, MPR300, lamp1, internalized transferrin and anti-MPR46 antibodies as well as neutral lipids stained by Nile red are shown in red. The distribution of each marker was tested in both cell lines and exhibited the same staining pattern.

We next analysed the localisation of TIP47 to certain organelles in HeLa and Cos cells via immunofluorescence using compartment-specific markers. These experiments showed that TIP47 did not colocalise neither with endogenous mannose 6-phosphate receptors nor with internalized antibodies against mannose 6-phosphate receptors (Figure 4.3.). Furthermore, TIP47 could not be colocalised with endosomal (internalised transferrin) and lysosomal (lamp1) markers, but decorated the surface of the lipid droplets, which were stained with a specific dye for neutral lipids (Nile red).

4.1.3 Localisation of tagged TIP47

To further confirm the association of TIP47 with lipid droplets and to avoid any doubts according to the specificity of the TIP47 reactive antibodies (Barbero et al., 2001), we generated recombinant TIP47 tagged at the amino- and carboxy- termini. To this end, total mRNA was prepared from HeLa cells, followed by RT PCR with TIP47 specific 5'- and 3'-primers. The obtained coding region of the HeLa cell-derived TIP47 cDNA was fully sequenced to confirm its identity.

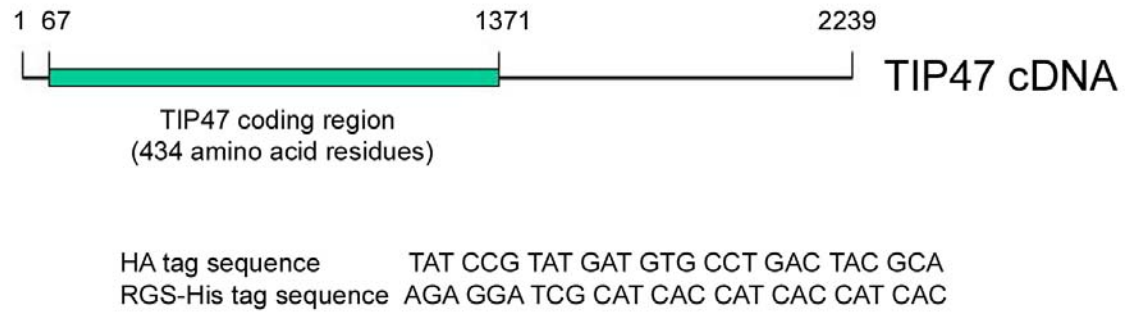

Figure 4.4. Schematic representation of TIP47 cDNA and sequences of the HA- and RGS-His- tags used. Numbers indicate the total length of the cDNA as well as length of the translated sequence. Only the TIP47 coding sequence was amplified and cloned with HA- or RGS-His- tags on N- or C-terminus into the mammalian expression vector pMPSV-EH. The nucleotide sequence of the HA-tag corresponds to YPYDVPDVA polypeptide, and the RGS-His-tag (later referred as His-tag) to the sequence RGSHHHHHH.

Sequencing revealed that the cDNA cloned matches a published sequence of the cargo selection protein TIP47 deposited by Diaz, E.D. and Pfeffer, S.R. (accession number AAC39751), which is slightly different from the TIP47 sequence deposed by Strausberg, R. (accession number AAH05818) harboring a conservative substitution: Ile56 was changed to Val. This change was independently observed by another

group (M. Rudolf, Department of Structural Biology, Göttingen, personal communications).

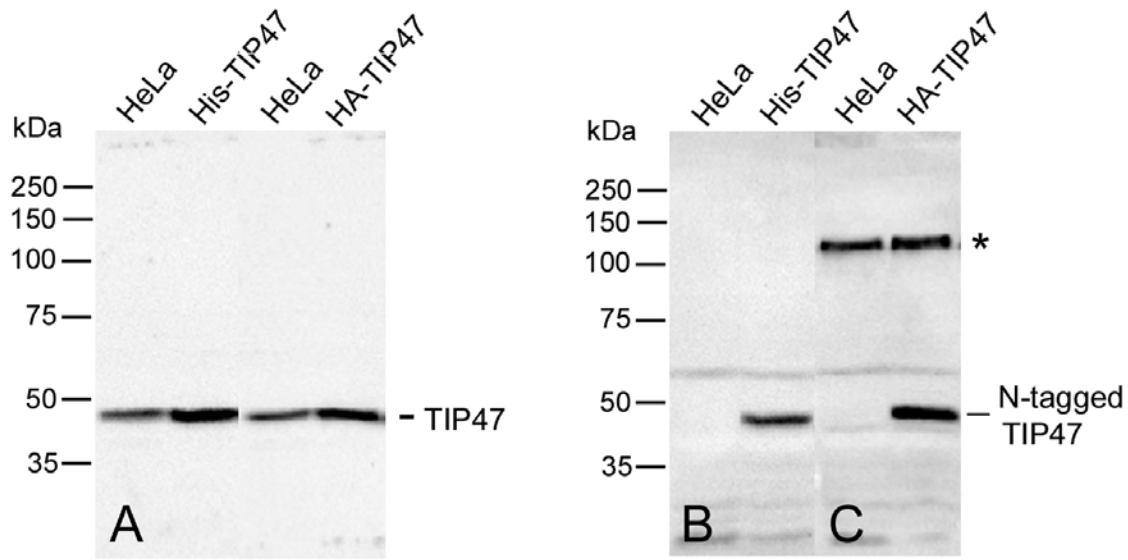

Figure 4.5. Lysates with equal amount of protein from untransfected and N-tagged TIP47 transfected HeLa cells were tested by Western blot analysis. In **A** the cell lysates were probed with rabbit anti TIP47 antibodies, in **B** with a monoclonal anti-His and in **C** with anti-HA antibodies. Both HA-and His-tagged TIP47 comigrate with endogenous protein. The asterisk (*) in C marks a protein which was unspecifically recognized by anti-HA antibodies.

The coding region of TIP47 cDNA was next used to generate tagged variants of the protein by fusing it either with the HA- or His- tag sequences in frame to the amino- or carboxy-termini (see 3.2.2.16 and Figure 4.4.). The derived tagged TIP47 was cloned into the pMPSV-EH vector and transiently expressed in HeLa cells under the control of the myeloproliferative sarcoma virus (MPSV) promoter (Artelt et al., 1988). Four days after transient transfection, total cells lysates of control HeLa cells and the transfected cells were prepared, subjected to SDS-PAGE, transferred onto nitrocellulose and tested with 3 antibodies: A) against TIP47, B) His- and C) HA-tags. As shown in Figure 4.5. a single band was labeled in transfected cells as well as in the control cells, when using a TIP47 antibody. Although one would have expected the tagged TIP47 to migrate at a slightly higher molecular weight, it could not be resolved in the Mini-gel system we were using. However, the extract of transfected cells exhibited a two-fold stronger signal, consistent with a moderate level of overexpression. Expression of the recombinant proteins was further confirmed by

using HA- and His-tag specific antibodies, which only stained a protein of the expected size in the lysates of transfected cells.

In contrast to efficient detection of N-terminally tagged TIP47 in western blotting (see Figure 4.5.), the C-terminal tagged variants were hardly visible (not shown), although they were suitable for the detection of TIP47 in immunofluorescence (not shown). As a consequence, all further studies involving tagged TIP47 were performed with the amino-terminal tagged TIP47.

After having verified the expression of the recombinant TIP47 by western blotting we performed immunofluorescence to localize the tagged protein together with endogenous TIP47 and the MPR46. Both TIP47 and the tagged protein colocalised to almost 100%, showing that the amino-terminal tag does not interfere with the localization of the fusion protein (Figure 4.6.). Moreover, the high degree of colocalisation demonstrated that the TIP47 specific antibodies stain the same structures, which are visualized by using tag-specific monoclonal antibodies. The experiment further revealed no colocalisation between TIP47 and MPRs, but staining of ring like structures that are identified as LDs by the incorporation of the lipophilic dye Nile red (see also Figure 4.26.).

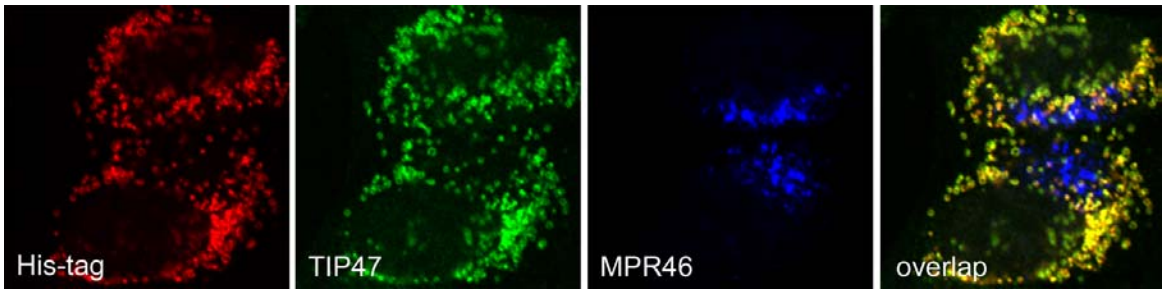

Figure 4.6. Tagged TIP47 does not colocalise with MPR46 in HeLa cells. Human TIP47 cDNA was cloned into the mammalian expression vector pMPSV-EH with His-tag and transiently transfected into HeLa cells. Two days after transfection, the cells were triple stained with rabbit anti-MPR46 (blue), guinea-pig anti-TIP47 (green) and mouse anti-His tag (red) antibodies and visualized with appropriate secondary antibodies. Endogenous TIP47 and the His-tagged protein exhibited colocalisation and were both well separated from the perinuclear staining of MPR46.

4.1.4 TIP47 cofractionates with isolated LDs

The association of TIP47 with lipid droplets was further revealed by an additional method well described in the literature – subcellular fractionation. After 12h

incubation with medium supplemented with 600µM oleic acid, the cells were harvested to prepare a postnuclear supernatant, which was further subjected to subcellular fractionation using a sucrose gradient. Indicative of LDs is that they float on top of such a gradient (Tauchi-Sato et al., 2002) from where they can be easily collected. As shown in Figure 4.7., a fraction of TIP47 could be detected in the floating material. The purity of the floating LDs was confirmed by blotting for other maker proteins, which were all well separated from the top fraction of the gradient. Thus, immunofluorescence as well as subcellular fractionation could unequivocally demonstrate the association of TIP47 with LDs.

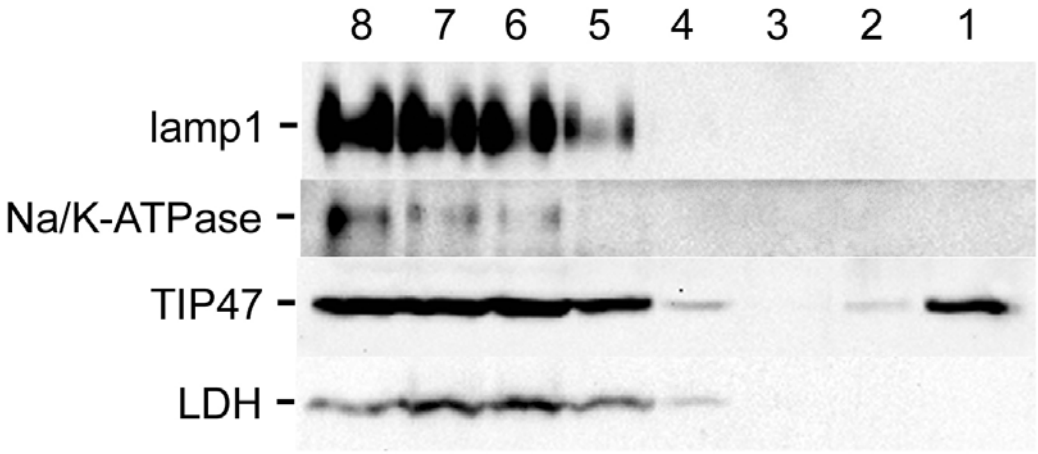

Figure 4.7. Endogenous TIP47 cofractionates with LDs in a sucrose gradient. HeLa cells were incubated 12h in medium supplemented with 600µM oleic acid and then fractionated as described above (3.2.3.1.4). Aliquots of each gradient fraction were run on 10% SDS-PAGE, transferred onto nitrocellulose membrane and probed with antibodies against TIP47 and markers of several compartments: lactate dehydrogenase (LDH) – marker of the cytosol; lamp1 – late endosomes/lysosomes; Na/K-ATPase – plasma membrane. The lipid droplet fraction was well separated from the markers of other compartments and contained TIP47. The LDs are known to float on top of the gradient and are collected in fraction 1. The western blot clearly showed that a fraction of TIP47 (16%) is associated with floating LDs.

4.1.5 TIP47 is not uniformly distributed on the LD surface

The immunofluorescence showed that most often TIP47 covers not the whole surface of the LDs but concentrates to certain “domains” of the organelle (Figure 4.8.). This observation may suggest that the LD membrane is organized into microdomains and it may indicate that the LD membrane is of complex organization.

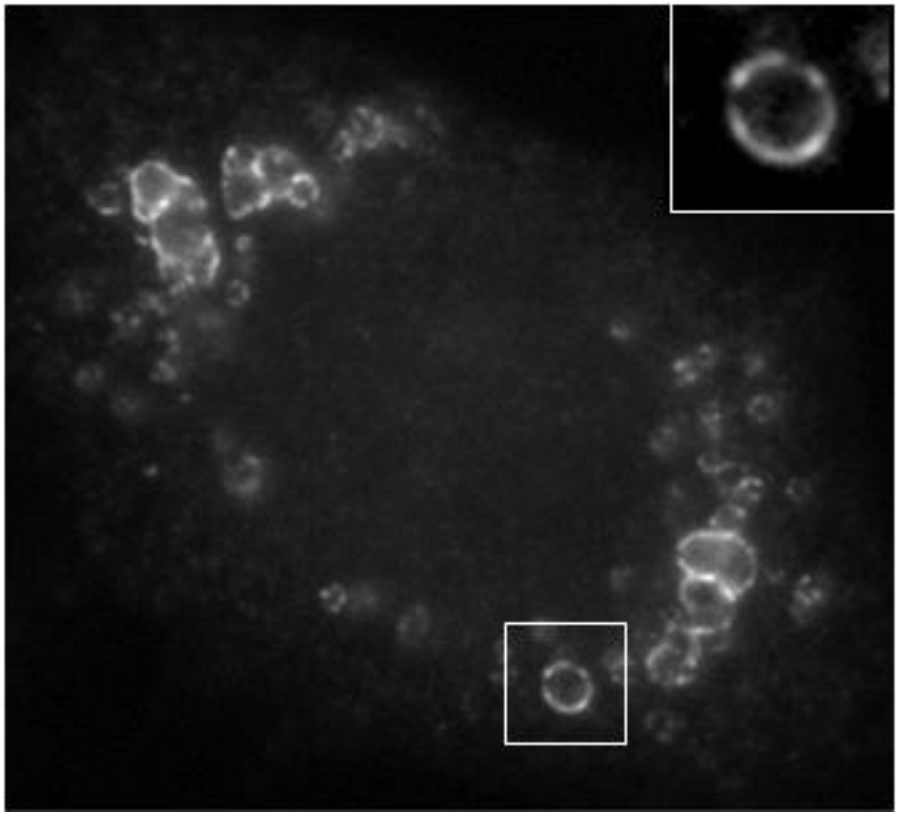

Figure 4.8. Distribution of TIP47 on the surface of lipid droplets. HeLa cell fixed with 3% PFA, permeabilised with 0.1% saponin and incubated with an antibody against TIP47 as described. Note that the protein is not randomly distributed and concentrated to certain “microdomains” (inset).

4.1.6 Time course of TIP47 recruitment to the LD

It was already shown by another group that the bulk of TIP47 is cytosolic and that upon accumulation of neutral lipids a fraction of the protein is recruited to the surface of the LDs (Wolins et al., 2001), however, nobody so far analysed the dynamics of TIP47 association with LDs. The time course of TIP47 recruitment to the organelles and its dependence on the nutritional status of the cell were studied in HeLa cells by conventional and quantitative immunofluorescence analysis. In order to estimate the fluorescence intensity of TIP47 positive LDs, 15 images were acquired for each time point in two independent experiments and the mean values of the fluorescence intensity were plotted.

We first analysed early events in LDs formation in HeLa cells preincubated overnight in starving medium (standard DMEM, supplemented with 2% FCS only). The preincubation leads to depletion of intracellular LDs as detected by Nile red and

TIP47 staining (Figure 4.12.). However, already 5-10 min after addition of 600µM oleic acid to the culture medium, the first TIP47 positive structures with an average diameter of 100-200nm appeared (Figure 4.9.).

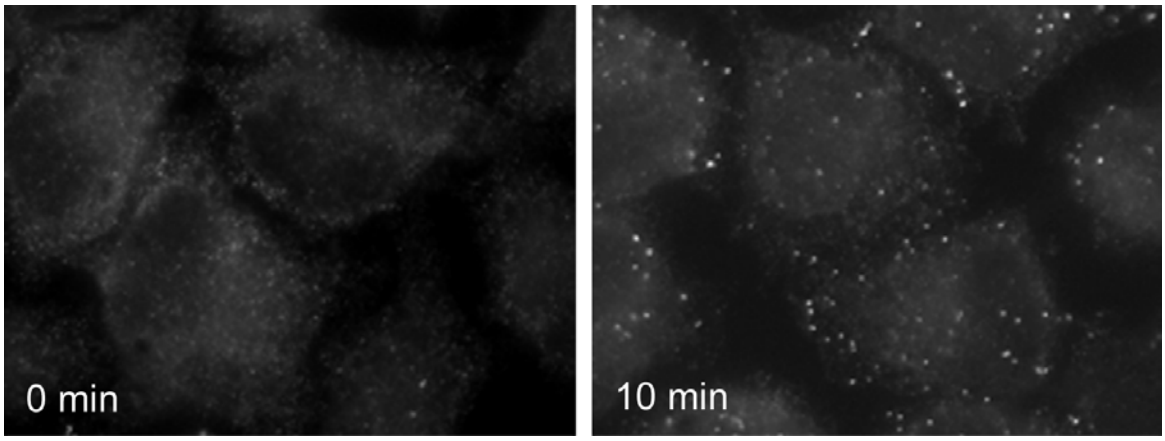

Figure 4.9. TIP47 translocates to the nascent lipid droplets early upon addition of oleic acid to the culture medium. HeLa cells were preincubated in presence of 2% FCS overnight and supplemented with DMEM containing 10% FCS and 600µM oleic acid. First TIP47-positive structures were observed after 5-10 min incubation. The staining observed in the left image is background fluorescence that becomes visible because the two images were overexposed to show the small and relatively faint TIP47 decorated structures.

After longer periods of incubation, the quantity of the structures increased, they became larger in size and were later brightly stained with Nile red, as illustrated in Figure 4.10. Remarkably, the TIP47 staining of the nascent lipid droplets was maximal within 4-6h after administration of oleic acid, while the incorporation of Nile red was delayed by several hours (see graph in Figure 4.10., next page).

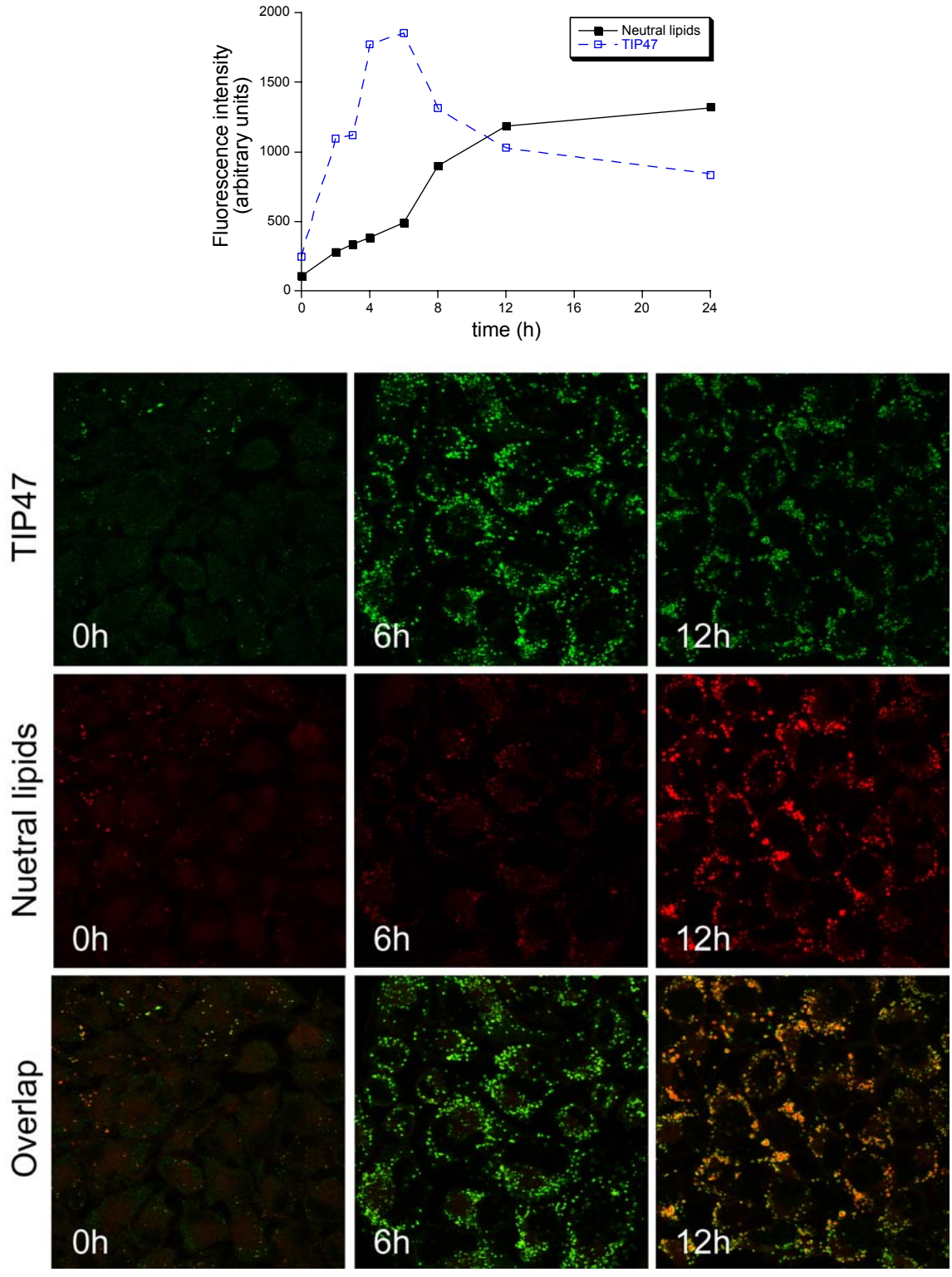

Figure 4.10. TIP47 recruitment to lipid droplets is maximal before the vast amount of neutral lipid is accumulated. HeLa cells were grown in normal medium (3.2.1.1) overnight and fixed or incubated in presence of 600μM oleic acid for 2, 3, 4, 6, 8, 12 or 24 h before indirect immunofluorescence (IIF) was performed. The quantitative data obtained by means of a Leica Confocal system are summarized in the graph, representative pictures are shown below. Note that the fluorescence intensity of TIP47-positive structures (shown in green) reaches its half-maximum within 2-3h upon stimulation of LDs formation, while the accumulation of neutral lipids (as indicates Nile red staining, shown in red) is delayed to 7h after feeding.

The next aspect of interest was the influence of the nutritional status of a cell on TIP47 recruitment to membranes. Cells were incubated under starving conditions (2% FCS) overnight before detection of TIP47 by immunofluorescence (Figure 4.11, A), or they were fed with 600µM oleic acid after starvation and then processed for IIF (Figure 4.11, B).

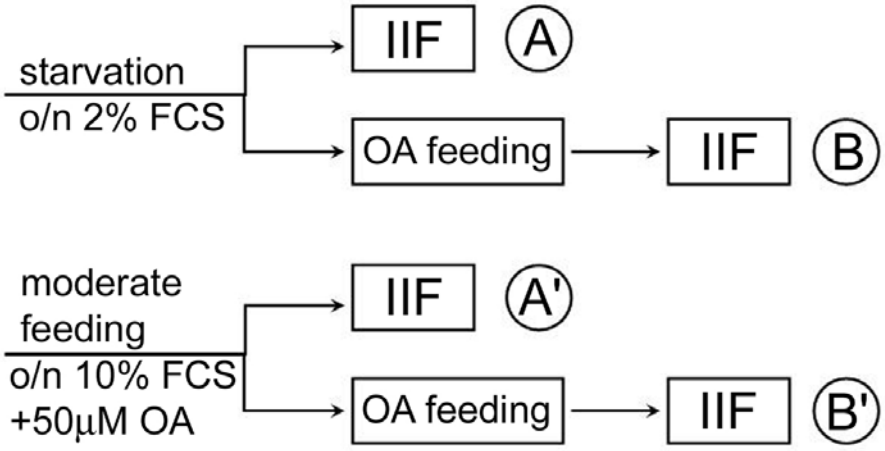

Figure 4.11. Influence of the nutritional status on recruitment of TIP47 to membranes. Outlined is the incubation protocol before TIP47 localisation was assayed by indirect immunofluorescence (IIF). Corresponding micrographs are shown in the next figure.

Alternatively, another batch of cells was incubated overnight under “moderate” feeding conditions (10% FCS + 50µM oleic acid) before they were processed for TIP47 staining (A’), or they were further fed with 600µM oleic acid before TIP47 detection (B’).

As shown in Figure 4.12., cells that were fixed immediately after starvation or moderate feeding both show a very low degree of TIP47 positive structures and almost no LDs as indicated by Nile red staining (compare A and A’). This similar pattern for TIP47 changed, when cells that were fed with oleic acid after starvation (Figure 4.12., B) are compared to those that received oleic acid after moderate feeding (Figure 4.12., B’). The later ones show a weaker TIP47 staining. This was also confirmed by a quantitative analysis of TIP47 fluorescence intensity (see graph in Figure 4.12.). Interestingly when cells were analysed that had been fed for longer periods of time (> 4h), the difference between starved and moderately fed cells became minimal, suggesting that only

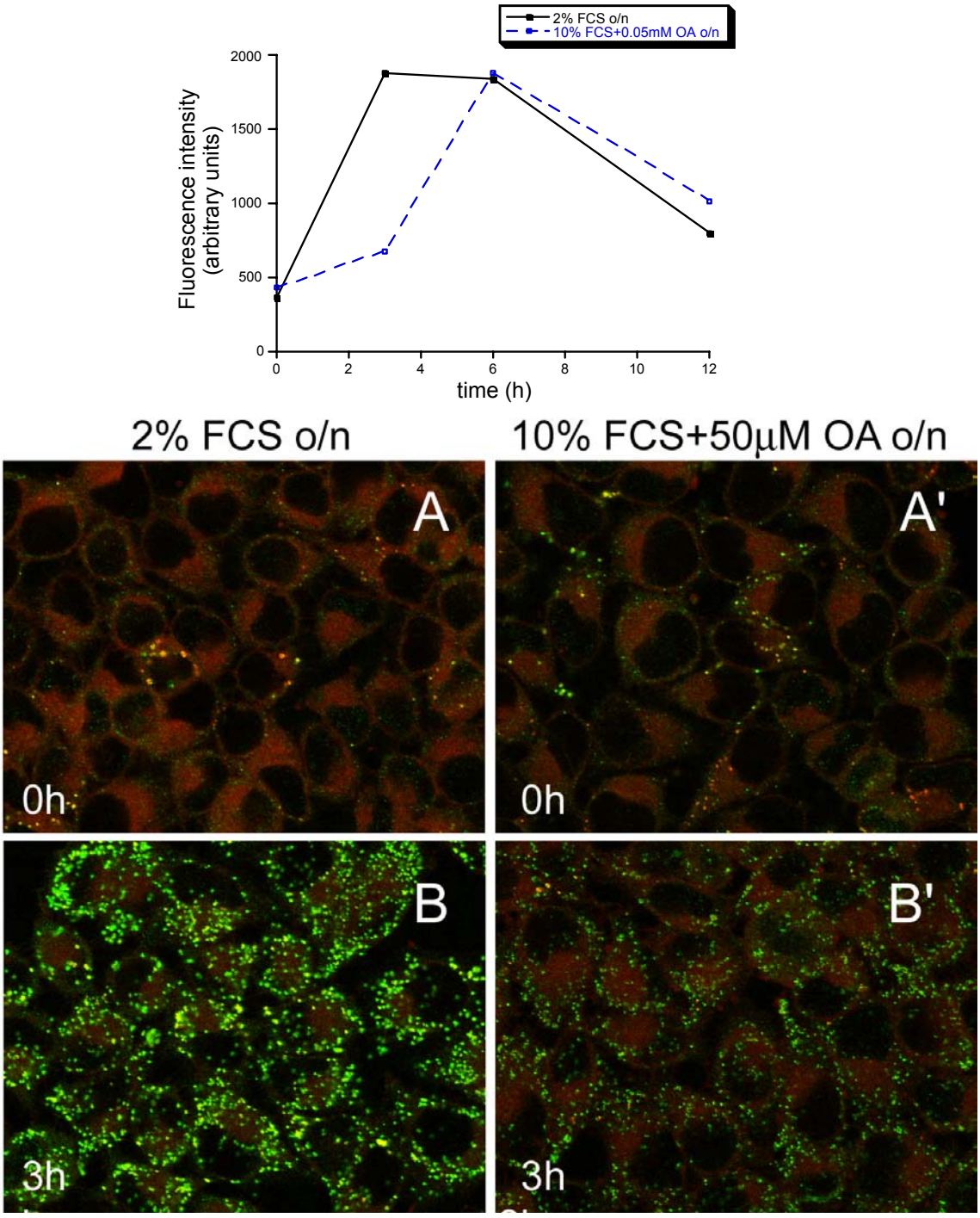

Figure 4.12. The time course of TIP47 recruitment to the lipid droplets depends on nutritional status of the cells. HeLa cells were incubated overnight in presence of 2% FCS or 10% FCS+50µM oleic acid, then supplemented with DMEM with 10% FCS and 600µM oleic acid and incubated 3, 6 or 12h, fixed and used for immunofluorescence analysis. The graph and photos above show that TIP47 translocates from the cytoplasm to the surface of the lipid droplets faster in starving cells and achieves its half-maximum in 1,5h, while in moderately prefeeded cells it occurs after 4h incubation with 600µM oleic acid.

shortly after administration of fatty acids, the cellular response with respect to the recruitment of TIP47 to LDs is dependent on nutritional status.

One could, for example, imagine that after feeding, TIP47 is primed somehow (phosphorylation or other posttranslational modification) to ensure rapid formation of new LDs after the cells receive new lipid. Alternatively, in a kind of negative feedback, feeding cells may lead to TIP47 modification that inhibit its rapid association with newly forming organelles. Another alternative is that not TIP47 by itself is the substrate that is modulated, but another factor associated with newly formed LD, which would mean that the effects on TIP47 are just secondary.

4.2 Towards the function of TIP47

The finding that TIP47 associates with the LD membrane early in the biogenesis of the organelle (see 4.1.6) is suggesting that LD formation involves the function of TIP47. However, TIP47 may also participate in the incorporation and/or exchange of material from the cytoplasm to LDs. As a starting point for a functional analysis, an RNAi approach has been chosen to suppress the expression of the protein, followed by the analysis of the affected cells.

4.2.1 Establishment of a TIP47 RNAi cell line

A TIP47 RNAi cell line was established as described in Materials and Methods (3.2.2.17). Shortly, two 19 nucleotide sequences flanked by AA-TT (starting at position 486 and 1435 of the full length TIP47 cDNA (shown in Figure 4.4) have been chosen to target human TIP47 mRNA by a plasmid based RNAi technique. The targeting sequence in direct and reverse orientation separated by a spacer (schematically shown in Figure 4.13.) were cloned into the plasmid pSHH, containing a U6 promoter and a neomycin resistance gene.

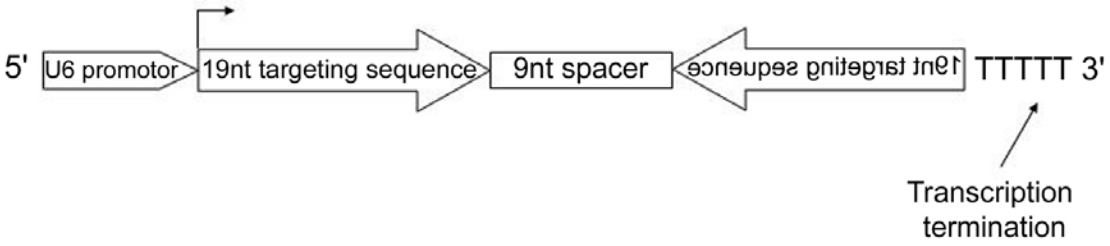

Figure 4.13. Schematic representation of the TIP47-specific primer sequence that generates a hairpin RNA in the cell. (From GeneSilenser™ Manual with modifications). Oligonucleotides were synthesized harboring the 19 nucleotides targeting sequences, a 9 nucleotide spacer and the targeting sequence in reverse orientation, flanked by a terminator of transcription (5T). They were cloned under the U6 small nuclear RNA promoter into the pSHH plasmid using XhoI and XbaI sites. The resulting vectors were stably transfected into HeLa cells in order to suppress the expression of TIP47.

The multicloning sites containing the RNAi constructs of the derived vectors were sequenced (3.2.2.18) followed by transfection into HeLa cells (3.2.1.5). HeLa clones resistant to 800µg/ml neomycin were picked and propagated. The level of TIP47 expression was then tested by western blot (Figure 4.14.). Only one of the two targeting sequences, namely CCCGGGGCTCATTTCAAAC, starting at position 1435 in the 3' nontranslated region of the mRNA was efficient in suppressing the TIP47 expression level in HeLa cells. Clone number 1, in which 92% of expression were suppressed, was used in further experiments to elucidate the TIP47 function.

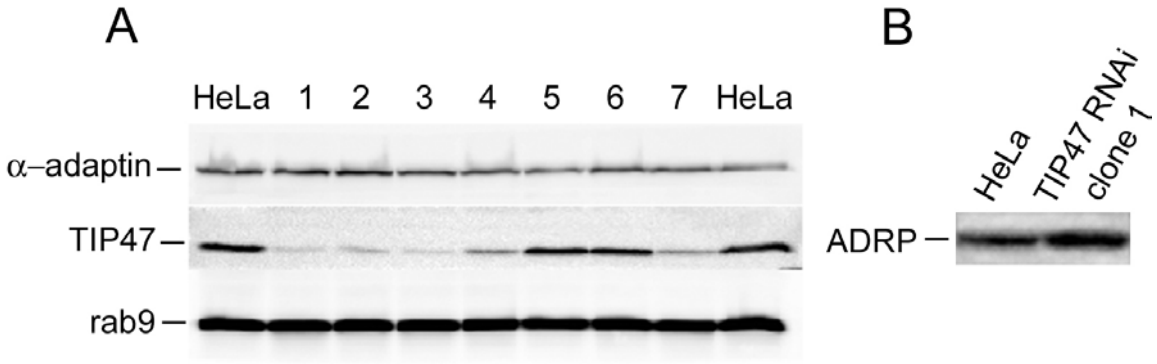

Figure 4.14. Lysates of control HeLa cells and TIP47 RNAi clones were analyzed by western blotting. 30µg of protein were loaded on each lane, electrophoretically separated and transferred onto nitrocellulose membrane. Primary antibodies used in the western blot are listed below. (**A**) Expression of TIP47 was efficiently and specifically suppressed in clones 1-3, while expression of other trafficking related proteins such as α-adaptin and rab9 was not affected. The different TIP47 RNAi clones are indicated with numbers. Clone number 1, expressing only 8% of normal amounts of TIP47 was used for further experiments. (**B**) Importantly, the expression of ADRP, the most closely related protein to TIP47 (43% identity) and associated with LDs – was not suppressed in TIP47 RNAi cells.

It is worth to mention that suppression of TIP47 was specific, since the expression of other trafficking related proteins (α-adaptin, rab9) and adipophilin (associated with LDs) was not affected.

Interestingly, the remaining small amount of TIP47 that is left in TIP47 RNAi cells localized to the surface of the LDs in contrast to HeLa cells, where it is mostly cytosolic, as shown by immunofluorescence analysis (see Figure 4.18.) and subcellular fractionation (Figure 4.15.). The consequence of this finding is discussed later in the text (see 5.1.).

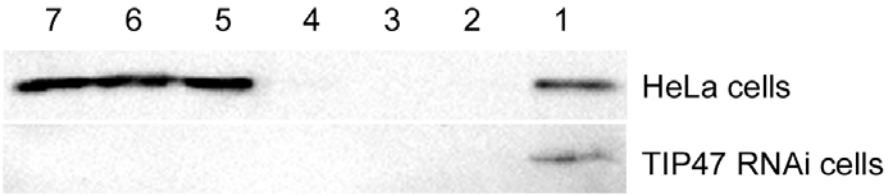

Figure 4.15. TIP47 is predominantly localized on the LDs in TIP47 RNAi cells. Control HeLa cells and TIP47 RNAi cells were incubated in presence of 600μM oleic acid for 12h and subjected to subcellular fractionation in a sucrose gradient. Fractions (indicated with numbers) were collected as outlined in 3.2.3.1.4. In contrast to the control HeLa cells, where the bulk of TIP47 is cytosolic with 16% of TIP47 cofractionating with LDs, in the TIP47 RNAi cells all TIP47 is associated with LDs upon subcellular fractionation. It is important to note that the western blot of the RNAi material had to be overexposed in order to detect any TIP47 signal.

4.2.2 Characterization of TIP47 RNAi cell line

4.2.2.1 MPRs transport is not affected by TIP47 RNAi

Since TIP47 was supposed to play a key role in recycling of MPRs from late endosomes to the TGN (Diaz and Pfeffer, 1998), several aspects of MPRs trafficking in HeLa and TIP47 RNAi cells had to be examined. First, the depletion of TIP47 did not affect the steady-state distribution of MPR46 as detected by immunofluorescence (see Figure 4.16.). It was also observed that MPR46 did not accumulate in an endosomal/lysosomal compartment in TIP47 RNAi cells under normal cell culture conditions (Figure 4.16.) or in presence of a lysosomal protease inhibitor (leupeptin 100μM, not shown).

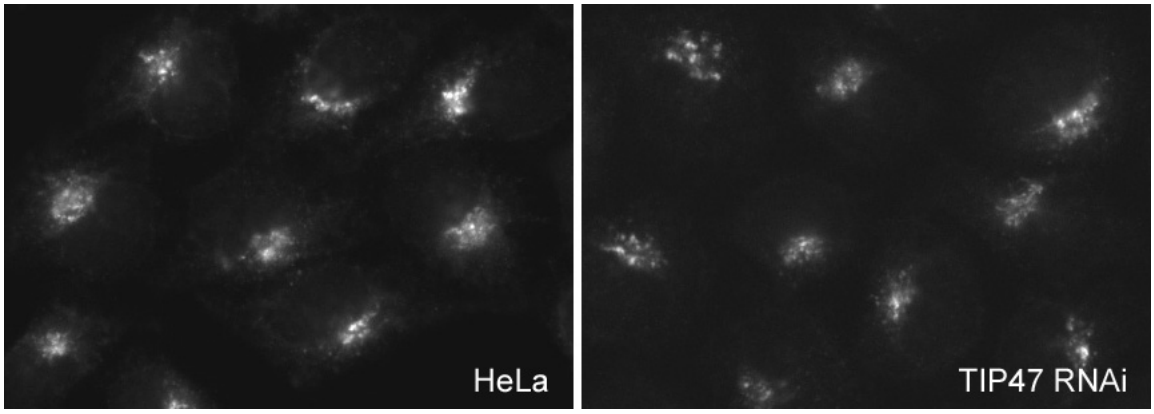

Figure 4.16. The steady-state distribution of MPR46 in TIP47 RNAi cells is not affected. Control HeLa cells and TIP47 RNAi cells were processed for immunofluorescence as described before. The staining pattern of MPR46 in RNAi cells shows no difference as compared to the control cells.

It is known that minor fractions of both MPRs are delivered to the plasma membrane, from where they are internalized and recycle back to the TGN. This transport can be visualized by an antibody uptake experiment during which antibodies that recognize the luminal domain of MPR46 or MPR300 are present in the medium. After 20min at 37°C, cells were fixed and further processed to visualize the internalized antibodies. As shown in Figure 4.17. anti-MPRs antibody transport to the TGN is equally efficient in control and RNAi HeLa cells, suggesting that the trafficking of MPRs is not affected by suppression of TIP47.

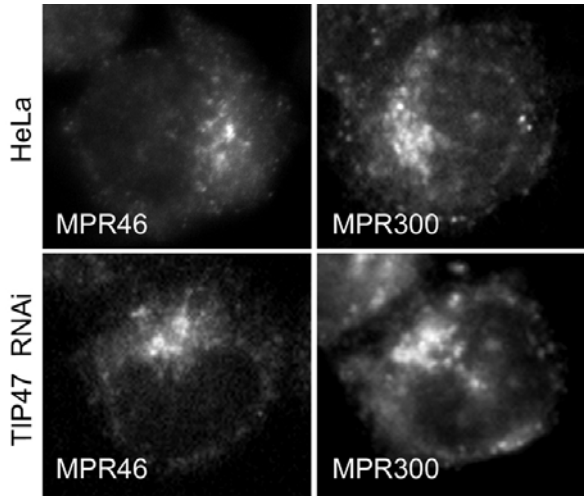

Figure 4.17. Internalized anti-MPRs antibodies are efficiently transported to the TGN region in both HeLa and TIP47 RNAi cells. Cells were incubated with anti-MPR46 or anti-MPR300 antibodies at 37°C for 20 min, fixed, permeabilised and incubated with secondary antibodies to visualize the internalised primary antibodies.

The half life of MPRs is strongly influenced by the efficiency of its retrieval from endosomes and transport back to the TGN (Rohrer et al., 1995). Therefore, we next tested the half life of MPR46 in HeLa and TIP47 RNAi cells in a pulse-chase experiment followed by immunoprecipitation of the receptor. Such experiments could not show any difference in the half life of the MPR46 between TIP47 RNAi and control cells (not shown). This result as well as the previous data strongly suggest that TIP47 has no role in the retrieval of MPRs from endosomes.

4.2.2.2 The effect of TIP47 RNAi on LDs

4.2.2.2.1 LD morphology is altered by TIP47 RNAi

As the previous results have shown the association of TIP47 with LDs it was now obvious to analyse the effect of the almost complete TIP47 suppression on various aspects of LD biogenesis, metabolism as well as the composition of the organelles.

A first observation was that the overall morphology of LDs formed in TIP47 RNAi cells after 12h incubation in presence of 600µM oleic acid had changed in comparison to HeLa cells. The appearance of LDs was assessed by immunofluorescence, LDs were stained for TIP47 and neutral lipids (Nile red). 15 confocal images were taken for each cell line after two independent experiments to analyse the size, number and amount of LDs per cell. It turned out that the amount and size of LDs were affected. The RNAi cells exhibited LDs of very variable size as compared to the control cells and their number was reduced in many cells (Figure 4.18.). This may indicate that either TIP47 is involved in the formation of the organelles but it could also point to function of TIP47 in the size-control of LDs.

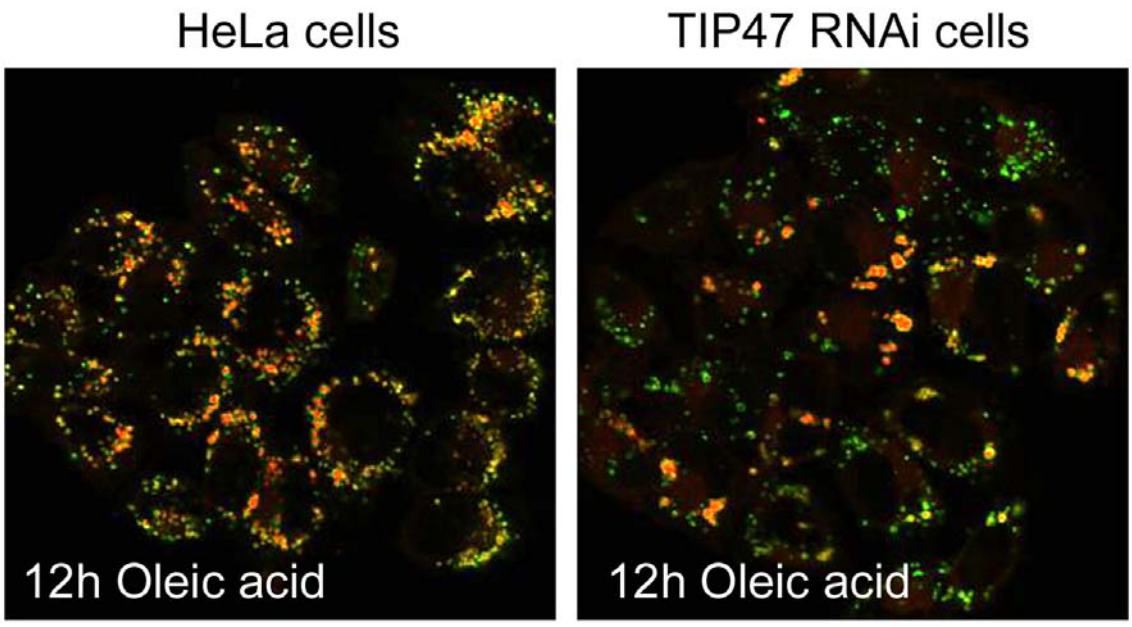

Figure 4.18. TIP47 RNAi cells form less uniform LDs than HeLa cells after 12h incubation in medium supplemented with 600μM oleic acid. The LD population formed by TIP47 RNAi cells is less uniform with respect to the organelle size and the LDs were less abundant than in the control. TIP47 is shown in green, neutral lipids are shown in red.

4.2.2.2.2 Fatty acid uptake and incorporation into LDs

Long chain fatty acids are efficiently incorporated into triacylglycerols (Guo et al., 2000), which are the major components of LDs in many cells. As these fatty acids are mostly imported from the cellular exterior we next elucidated whether TIP47 RNAi had any effect on the uptake of long chain fatty acids and their incorporation into LDs. Three species of ^{3}H- or ^{14}C-labeled fatty acids, saturated (palmitic), mono-unsaturated (oleic) and polyunsaturated (arachidonic) were added to the cells under three experimental conditions:

a) Radioactively labeled fatty acids were added to cells in suspension at a concentration of 20μM in PBS to estimate the efficiency of the fatty acids uptake over short time periods (5-30 min);
b) Radioactively labeled fatty acids were added to adherent cells at 20μM in the medium and incubated for periods ranging from 10 min to 12h;
c) Radioactively labeled fatty acids were diluted with the corresponding unlabeled fatty acids and added to the standard cell culture medium at 600μM and incubated with the cells for up to 8h.

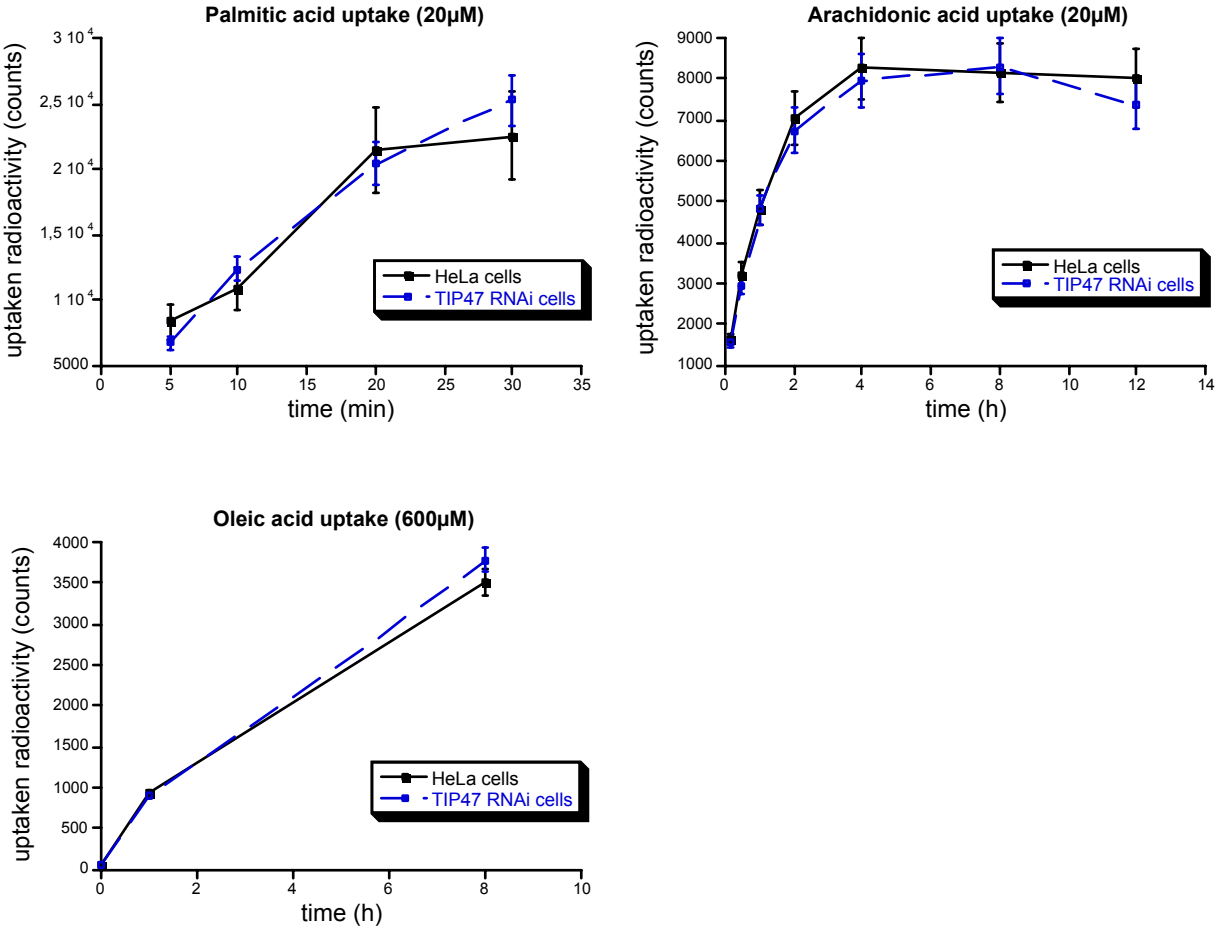

Figure 4.19 HeLa and TIP47 RNAi cells take up equal amounts of long chain fatty acids from the medium. The uptake of palmitic (saturated), oleic (mono-unsaturated) and arachidonic (polyunsaturated) acids in concentrations of 20µM or 600µM was monitored for different periods of time. All experiments were performed in duplicates (with oleic acid) or in triplicates (with palmitic and arachidonic acids), the incorporated radioactivity was normalized to the amount of protein.

After the incubation with fatty acids the cells were washed twice with PBS, harvested and homogenized, followed by determination of protein concentration and counting of the amount of incorporated radioactivity.

All these experiments showed that TIP47 RNAi cells take up normal amounts of either saturated or unsaturated long chain fatty acids under all experimental conditions tested.

As it was mentioned before, long chain fatty acids taken up by cells serve as substrates for synthesis of triacylglycerols, stored in LDs. After having shown that the uptake of fatty acids is not affected by TIP47 RNAi we next analysed the appearance of the radioactively labeled fatty acids in LDs. The LDs were purified by subcellular

fractionation as described above and analysed for incorporated radioactivity. In contrast to the unaffected uptake of fatty acids into the cells these experiments revealed 50% reduction in the incorporation of radiolabeled oleic acid into LDs (Figure 4.20). However, the current data does not allow to draw the conclusion that TIP47 is directly involved in the incorporation of radiolabeled material into LDs.

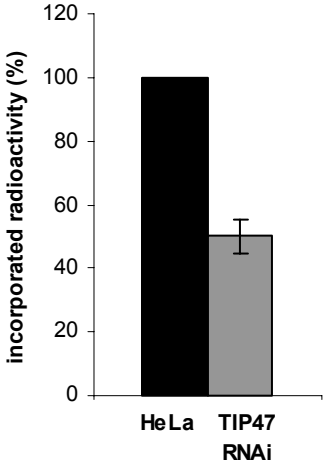

Figure 4.20. Oleic acid incorporation into LDs of TIP47 RNAi cells is reduced. HeLa and TIP47 RNAi cells were grown for 24h and then incubated during 12h in presence of 600µM cold oleic acid and 200nCi of ^{14}C-oleic acid, harvested and fractionated. The amount of radiolabeled oleic acid in LDs was normalized to the cellular protein and plotted. The standard deviation of three experiments is shown.

4.2.2.2.3 Phospholipid and protein composition of lipid droplets

The loss of TIP47 expression may not only cause subtle effect on the incorporation of certain contents into the organelles, it is of course also possible that the overall LD associated protein and/or lipid composition is changed. To search for such changes, cells were incubated in presence of 600µM oleic acid for 12h to induce LD formation. Subsequently, the cells were harvested and subjected to subcellular fractionation to collect the LDs. The associated lipids and proteins were separated by methanol-chloroform extraction (see 3.2.3.1.4). A fraction of the extracted lipids was then directly applied to Maldi-MS. Such analysis only allows to detect major changes in the phospholipid composition while subtle changes in single species of phospholipids as well as other lipids will not be identified. As shown in Figure **4.21**. the Maldi-MS spectra look almost identical, indicating no major changes in the phospholipid composition of LDs from TIP47 RNAi cells. I of course aware of the fact that only a

more sophisticated MS analysis is needed to elucidate effect on single phospholipid species, which cannot be ruled out by the method used.

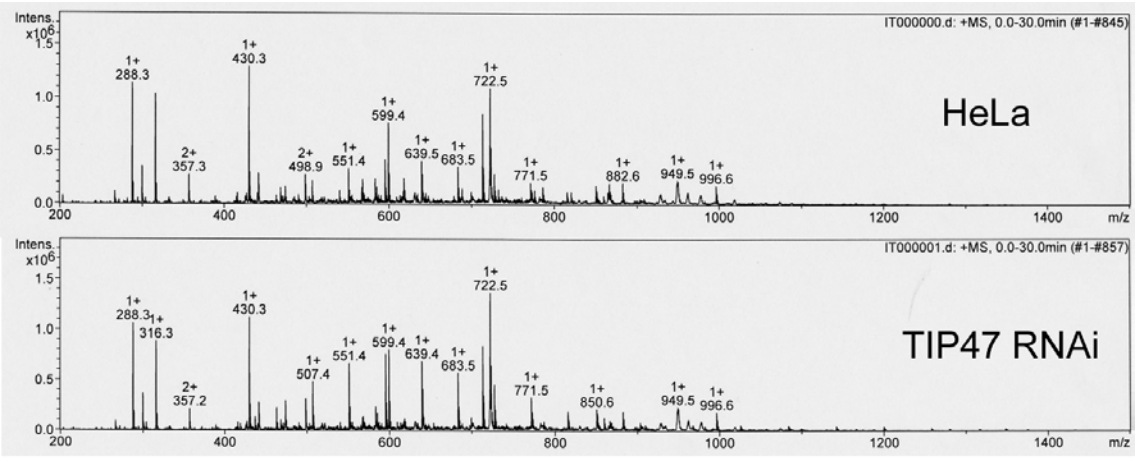

Figure 4.21. No major differences were observed in the phospholipid composition of isolated lipid droplets of HeLa and TIP47 RNAi cells by means of Maldi-MS. LDs isolated from TIP47 RNAi and HeLa cells by sucrose gradients were subjected to methanol-chloroform extraction. The derived lipids were collected and used for Maldi mass spectrometry.

The extracted proteins were separated by SDS-PAGE and silver stained (Figure 4.22.). Only one major difference in the protein pattern between control and TIP47 RNAi cells was detectable, - a substantial reduction of a protein with a molecular weight below 50kD. MS-analysis identified this protein as TIP47 (see A in Figure 4.22). This result also confirms the specificity of the RNAi since only the target protein is affected in its expression.

Another unexpected finding was that TIP47 belongs to the most prominent bands of the resolved LD associated proteins. Very recently, other groups have also started to analyse the proteom of LDs (Athenstaedt et al., 1999; Liu et al., 2003). In contrast to the attempt of Anderson and coworkers (2003) who washed their purified LDs very extensively, our LDs derived from subcellular fractionation were directly subjected for SDS-PAGE. Interestingly, TIP47 is not associated with such extensively washed LDs, suggesting that washing is useful to reduce some contaminating proteins but also harbors the risk of loosing proteins only weakly associated with the LD membrane.

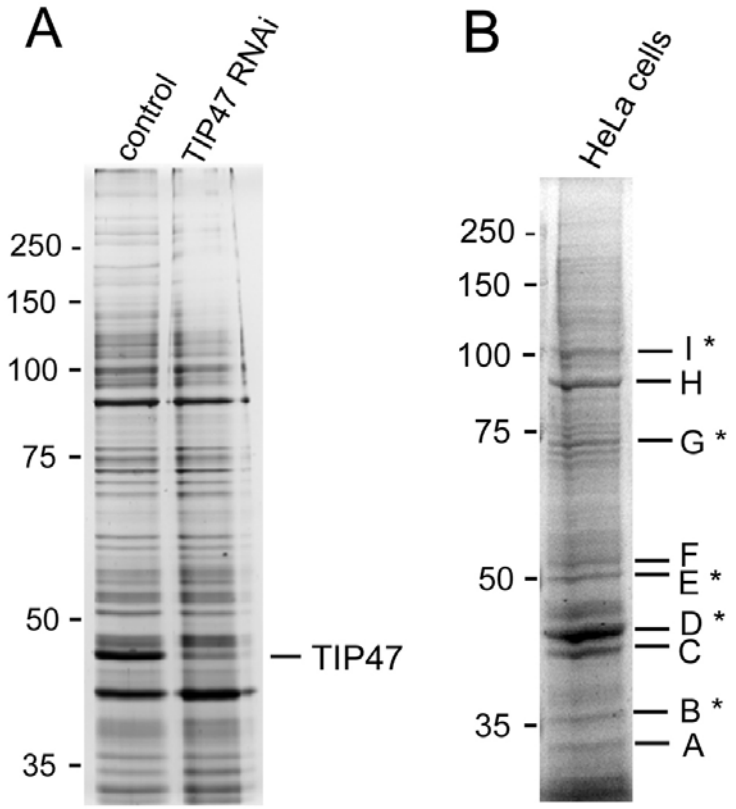

Figure 4.22. (**A**) TIP47 RNAi does not alter the typical LD protein composition. LDs collected after subcellular fractionation were extracted with methanol-chloroform and then subjected to SDS-PAGE. Equal amounts of protein were loaded. TIP47, one of the major proteins visible in the control LDs is reduced to a faint band in RNAi cells, showing the specificity and efficiency of the approach. (**B**) LDs of 6 15cm dishes HeLa cells were collected, resolved by SDS-PAGE and stained with coomassie. Some of the protein bands were subjected to Maldi-MS identification. Letter A indicats glyceraldehyde-3-phosphate dehydrogenase, B - NAD(P) dependent steroid dehydrogenase like protein, C - β-actin, D – TIP47, E - adipophilin, F - tubulin β5, G - fatty acid (long chain) CoA ligase 3, H - heat shock protein 1-β of 90kD, I - cytosolic calcium-dependent phospholipase A2 (group IVA). Asterisks (*) mark known LD associated proteins.

A Coomassie blue stained SDS-PAGE of HeLa derived LDs is shown in Figure 4.22. B. From this gel we could identify the following proteins: 1) precursor of the heat shock protein gp96; 2) cytosolic calcium-dependent phospholipase A2 (group IVA); 3) α-actinin 4; 4) heat shock protein 1-β of 90kD; 5) fatty acid (long chain) CoA ligase 3 (known as well as acyl-CoA synthetase 3); 6) tubulin β5; 7) adipophilin; 8) β-actin; 9) NAD(P) dependent steroid dehydrogenase like protein and glyceraldehyde-3-phosphate dehydrogenase. Although the lack of washing results in the detection of proteins not typical for LDs such as actin and tubulin, most other proteins were indeed already identified as LDs-associated showing the rational of our approach.

4.3 TIP47 secondary structure and modifications

4.3.1 Database search

Despite the knowledge of the exact aminoacid composition of the full-length TIP47 protein, almost nothing is known about structural features or about putative post-translational modifications, no it is known how TIP47 associates with the LD membrane. However, these aspects are of great importance in order to understand how TIP47 is recruited to membrane, whether it has binding partners and how its function is regulated.

The analysis of TIP47 using the PredictProtein site resulted in the prediction of a diffuse globular structure without defined domains, such as membrane spanning domains. The only structural features of TIP47 known so far are putative coiled-coil regions in the C-terminal half of the protein and the N-terminal PAT domain (Figure 4.23.). The latter is of high homology to sequences also found in perilipin and adipophilin (PAT = perilipin, adipophilin, TIP47). The PAT domain of TIP47 containing the residues 22-116 share 60% identity with adipophilin and is to 38% identical and 60% similar to the amino-terminal region of human perilipin A (Garcia et al., 2003).

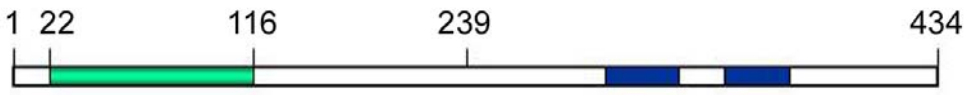

Figure 4.23. Known structural features of TIP47. The protein is thought to contain three main parts the N-terminal PAT-containing domain (1-116, PAT domain is shown in green), a fragment homologous to the adipophilin LD targeting domain (117-239) and the C-terminal half containing putative coiled-coil regions (blue).

We also searched for putative lipid anchors in TIP47 which could mediate interaction of TIP47 with the lipid droplets. According to the Prosite documentation palmitoylation can be predicted for Cys (Ser, Thr, Lys) in any position. TIP47 possesses 3 Cys residues (39, 60, 341) and each of them could be potentially palmitoylated. No C-terminal GPI-anchor or N-terminal myristoylation sites were found in the full length TIP47 a/a sequence by Prosite, big-PI Predictor v. 1.5 program and MYR Prediction Server.

The Prosite search resulted in the prediction of a number of potential Protein kinase C (PKC) and Casein kinase II (CK2) phosphorylation sites (16 Ser and 7 Thr in total) throughout the entire TIP47 sequence. By using the NetPhos 2.0 Server, 18 Ser, 7 Thr and 3 Tyr potential phosphorylation sites were predicted.

Two SUMO attachment sites with high probability (K195, score 0.6722 and K283, score 0.7556) were predicted by SUMOplot&trade Prediction Development by Abgent. The functional significance of this prediction is however totally unclear and awaits experimental proof.

4.3.2 TIP47 is a phosphoprotein

Phosphorylation is the most frequent protein modification regulating the function and intracellular translocation of proteins. However, it is not known so far whether TIP47 is a phosphoprotein. Since TIP47 contains many predicted phosphorylation sites and since its recruitment to the LD and/or its recycling into the cytosol, as well as its function might be regulated by phosphorylation, we analysed the potential incorporation of [^{32}P]-phosphate into TIP47. In a preliminary experiment, HeLa cells were labeled with [γ^{32}P]-ATP under three conditions: under low fatty acid concentration (5% dFCS), high (5% dFCS+600µM oleic acid) fatty acid conditions;

and thirdly cells were preincubated with 600µM oleic acid in standard medium and labeled in DMEM supplemented with 5% dFCS.

Immunoprecipitation of TIP47 was followed by SDS-PAGE of the precipitated material and autoradiography. Unfortunately, the autoradiograph was quite dirty with high background. However, a band corresponding to the molecular weight of TIP47 was labeled in all three samples with varying intensities. ^{32}P-labeling increased by 66% in cells incubated in the presence of 600µM oleic acid as compared to cells labeled under low FAs conditions, and decreased by 38% in cells preincubated with 600µM oleic acid and labeled under low FAs conditions (Figure 4.24).

This very preliminary finding suggests that human TIP47 is phosphorylated in vivo and that its phosphorylation status may depend on the availability of fatty acids for the cells. This result however has to be confirmed by additional experiments and is just considered as a first hint.

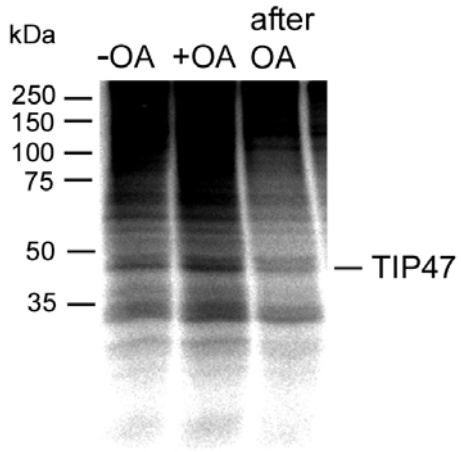

Figure 4.24. Phosphorylation of TIP47 in vivo. TIP47 was immunorecipitated from HeLa cells labeled with ^{32}P under low fatty acid conditions (- OA), under high fatty acid concentration in the medium (+ OA) and under low fatty acid conditions after preincubation with 600µM oleic acid. TIP47 phosphorylation was stimulated by high concentrations of oleic acid (OA) and may reflect a desensibilisation to the stimulation by the addition of fresh DMEM with 5% dFCS.

4.4 The LD targeting determinats of TIP47

The targeting to the LD membrane of proteins that belong to the PAT-family is unclear. To exploit this important aspect of TIP47, the protein was arbitrary divided into 3 parts (Figure 4.23.):

1) An N-terminal region including the PAT domain (residues 1-116);
2) A sequence homologous to the ADRP-LD recruiting region (McManaman et al., 2003) (residues 117-239);
3) A C-terminal part including two putative coil-coiled regions (residues 240-434).

According to the division of the protein, 5 HA-tagged variants of TIP47 were cloned, each containing large deletions on N- and/or C-termini (Figure 4.25.).

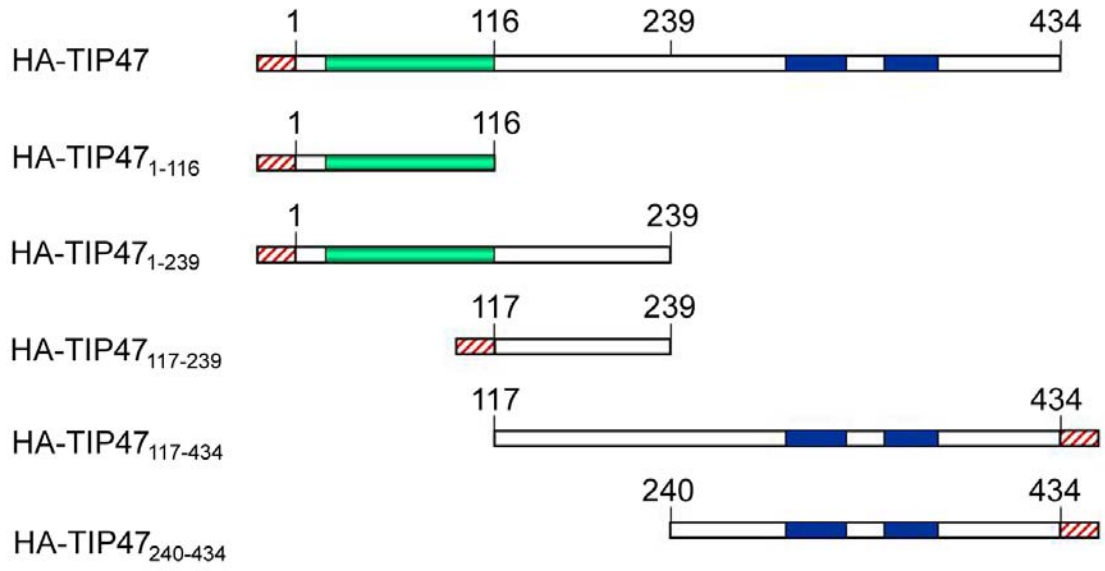

Figure 4.25. Schematic representation of tagged TIP47 variants. The colours used to indicate domains within TIP47 are the same as in Figure 4.23, HA-tag is shown in red. The lower 5 bars represent the HA-tagged TIP47 containing large deletions in the C-terminal part (HA-TIP47$_{1-116}$, HA-TIP47$_{1-239}$), in the N-terminal region (HA-TIP47$_{117-434}$, HA-TIP47$_{240-434}$) and a variant in which only the middle part was left (HA-TIP47$_{117-239}$). Five TIP47 truncation variants were tagged on the amino- or carboxy-terminus, as indicated in the figure.

All five TIP47 variants as well as the tagged full length protein were expressed in HeLa cells and their distribution was analysed by indirect immunofluorescence using anti-HA-tag specific antibodies after overnight incubation of the transfected cells in the presence of 300-600μM oleic acid (Figure 4.26.). Only the full length HA-TIP47 and HA-TIP47$_{1-239}$ were associated with LDs in all of the expressing cells, while HA-TIP47$_{117-434}$ localised to LDs in a small number of cells (~5%). All other variants showed a diffuse distribution.

This analysis is therefore suggesting that the targeting of TIP47 to LDs requires the N-terminal half of the protein, while the C-terminal part is not involved. It is however

important to note, that such large deletions may severely alter the structure of the protein, thereby affecting the recruitment to LD membrane. Thus further studies using much smaller deletions will be needed to narrow down the exact sequence that mediates LD membrane association.

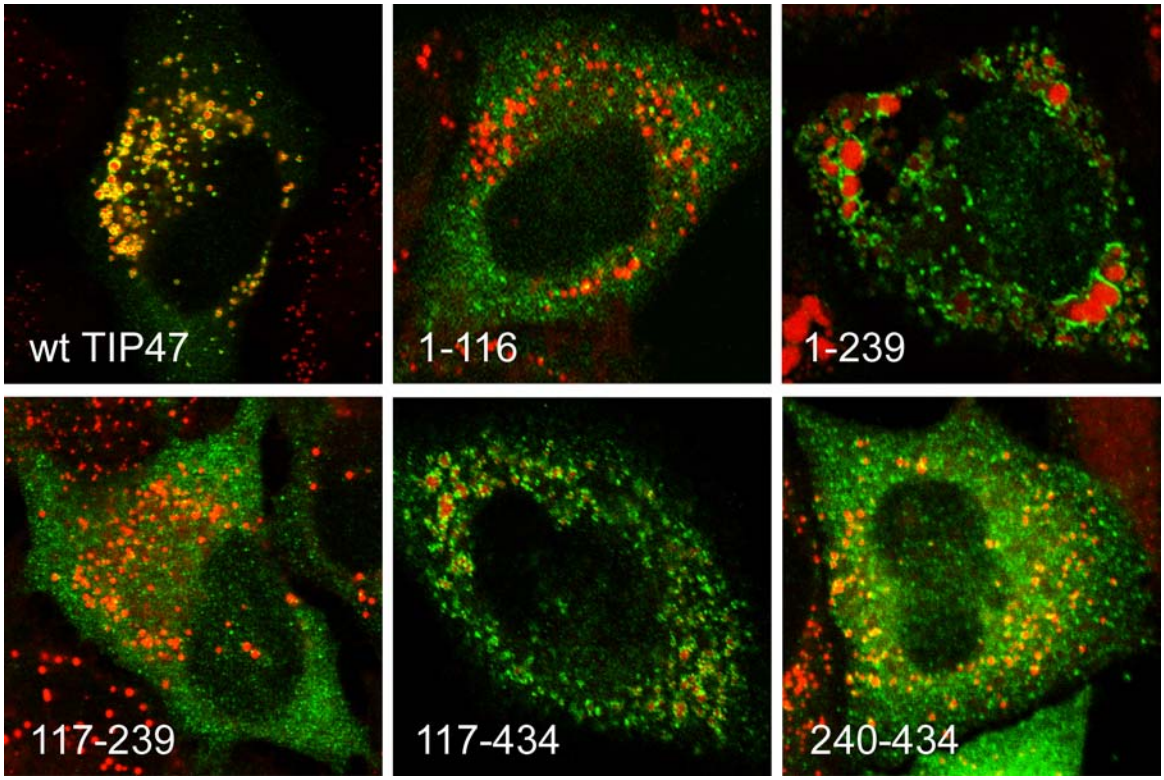

Figure 4.26. Distribution of TIP47 fragments in HeLa cells. One day after transient transfection with full length HA-tagged TIP47 or with one of the 5 TIP47 truncation variants, HeLa cells were incubated 12h in presence of 300 or 600µM oleic acid and processed for immunofluorescence. Three of the tagged proteins, *HA-TIP47*$_{1-116}$, *HA-TIP47*$_{117-239}$ and *HA-TIP47*$_{240-434}$ exhibited a diffuse localization. *HA-TIP47*$_{117-434}$ was associated with LDs in only about 5% of the transfected cells, while full length HA-TIP47 and *HA-TIP47*$_{1-239}$ were recruited to the LDs in all transfected cells. The HA-tag is shown in green, neutral lipids are shown in red.

5 Discussion

5.1 TIP47 does not participate in MPRs trafficking

TIP47 is supposed to be one of the candidate proteins that functions as a cargo selection devise for retrieval of MPRs from late endosomes. The idea is based on seven publications of the Suzanne R. Pfeffer's group. They analysed different aspects of TIP47 including its binding to MPRs and to the small GTP-ase rab9 (Barbero et al., 2002; Carroll et al., 2001; Diaz and Pfeffer, 1998; Hanna et al., 2002; Krise et al., 2000; Orsel et al., 2000; Sincock et al., 2003). Originally, TIP47 was found in a yeast two-hybrid system as a protein interacting with MPRs cytoplasmic tails as a bait (Diaz and Pfeffer, 1998). The work of the group suggested that TIP47

1) binds cytoplasmic domains of both MPRs in vitro;
2) colocalizes with MPR300 (immunofluorescence analysis);
3) is required for recycling of MPR46 from endosomes to the TGN in an in vitro transport assay;
4) is important for MPR300 stability in cells, where TIP47 expression was suppressed by antisence oligonucleotides;
5) specifically binds the FW motif in the MPR46 cytoplasmic domain. This motif was characterized by others as a lysosomal avoidance signal (Schweizer et al., 1997).

However, several recent publications have provided evidence for another function of TIP47 apart from a role in MPRs recycling. Wolins et al. (2001) showed that TIP47 does not colocalize with MPR46, but decorates the surface of LDs in HeLa cells. The results were questioned by S.R.Pfeffer but confirmed by two other independent groups (Miura et al., 2002; Than et al., 2003). In more detail, Pfeffer and colleagues (Barbero et al., 2001) claimed that TIP47 was visible on LDs due to low specificity and cross-reactivity of the antiserum used by Wolins et al., (2001). The authors insisted that the antiserum recognizes two additional proteins, adipophilin and an unknown protein in HeLa cell, and that usage of affinity purified antiserum against TIP47 does not result in staining of LDs in indirect immunofluorescence. This assertion is however very questionable since Wolins et al. (2001) tested the

antiserum used and found no cross-reactivity in western blotting and immunoprecipitation.

In the next publication on the issue, GFP-TIP47 fusion protein was found to concentrate around LDs in vitro and proposed to be a multifunctional protein, which has a potential to participate in both MPRs transport and LD formation (Miura et al., 2002). However, more convincing data were published just recently by Than et al. 2003, who detected TIP47 on LDs using a different antibody, which specificity to TIP47 was unequivocally confirmed by immunoprecipitation followed with MALDI-MS.

Another issue of uncertainty was the question of how TIP47 is recruited to membranous organelles from the cytoplasm. It was postulated that TIP47 is recruited to membranes due to its binding to MPRs cytoplasmic domains and that the interaction appears to be hydrophilic, as TIP47 could be released from membranes by 1M KCl (Diaz and Pfeffer, 1998). However, the same authors later showed that TIP47 binding to both MPRs is of hydrophobic nature (Diaz and Pfeffer, 1998; Orsel et al., 2000).

In the beginning of this work we wanted to use TIP47 as a marker protein of a special population of endosomes that are part of the MPR trafficking routes. However, the first simple intracellular localization of TIP47 via immunofluorescence raised doubts according to the published data.

To avoid any accusation in producing artefacts due to low specificity or cross-reactivity of any anti-TIP47 antibodies used, we tested our antibodies in Western blotting and immunoprecipitation. Further, we cloned TIP47 fusion proteins with HA- and His- tags. We found out that both antibodies (donated by S.R. Pfeffer or purchased from Progen) recognize a single protein in HeLa cell lysates in Western blotting and immunoprecipitation (Figure 4.1.) and give a signal perfectly overlapping with anti-HA or His antibodies when used in immunofluorescence on cells expressing tagged TIP47 (Figure 4.6.).

After having confirmed the specificity of our antibodies against TIP47, we analyzed the distribution of endogenous and tagged TIP47 by means of immunofluorescence and cell fractionation followed by western blot. Both endogenous and tagged TIP47 were indeed found to surround LDs stained with Nile red (Figure 4.3., Figure 4.26.). TIP47 did not colocalize with both MPRs nor with markers of endosomes and

lysosomes (Figure 4.3., Figure 4.6.) confirming that TIP47 is a LD associated protein (Wolins et al., 2001; Miura et al., 2002; Than et al., 2003). At the same time about 16% of total cellular amount of TIP47 were found in association with LDs upon lipid loading and subcellular fractionation (Figure 4.7.). Remarkably, TIP47 does not cofractionates with fractions devoid of LDs in TIP47 RNAi cells (Figure 4.15.), indicating that in these cells it is virtually absent in the cytosol and does not associate with membranous organelles apart from LDs.

In addition when the RNAi cells were analysed with respect to effect on MPRs we found out that MPRs trafficking was not affected at all. MPRs distribution (Figure 4.16.), recycling of anti-MPRs antibodies (Figure 4.17.) and the half life of MPR46 (not shown) were not affected in TIP47 RNAi cells. G.R. Medigeshi in the group of P.Shue could also show that a lysosomal enzyme cathepsin D, dependent in its trafficking on MPRs, was not missorted and that TIP47 is not required for MPR46 recycling in an in vitro transport assay (Medigeshi and Schu, 2003). Moreover, we were also informed by another group that it was not possible to reproduce the in vitro interaction between TIP47 and rab9 (Markus Rudolf, unpublished data).

Taken together, the data presented here as well as published earlier (Wolins et al., 2001; Miura et al., 2002; Than et al., 2003; Medigeshi and Schu, 2003) demonstrate that TIP47 is a constituent of LDs and has no role in MPRs transport.

5.2 LDs biogenesis

The formation of LDs in mammalian cells is generally assumed to be the result of the biosynthetic activity of ER localized enzymes and pooling of triacylglycerol or other lipids down to LDs (Murphy, 2001). In contrast to mammalian LDs, which were not extensively studied with respect to their biosynthetic activity and protein composition, yeast LDs are well established biosynthetically active organelles (Murphy, 2001; Sorger and Daum, 2003). However, the number of publications describing the protein composition of mammalian LDs (e.g. (Liu et al., 2003)) and their enzymatic activities (Yu et al., 2000) is growing.

In mammalian cells, LD biogenesis as well as the regulation of its function are only poorly understood. Some models have been proposed, which in many aspects

remain illusive why I want to point a few important aspects that are related to my current work.

The first question is whether LDs are organelles? Are they indeed surrounded by an individual membrane, or conversely are they in continuity with the ER? The question seems to be simple to answer, however, some of the methods were not correctly used in the past and therefore many studies addressing the aspect of LD organelle identity are of very limited value. Immunofluorescence studies are for example beyond the limit of resolution to allow a clearcut answer. The tubular ER network emerges into almost all areas of the cell and thus almost all other cellular structures are in very close apposition to the ER.

One would immediately try to make use of electron microscopy (EM). However sample preparation for EM is known to cause artefacts such as breakage of small connections between organelles. If therefore an ultrastructural analysis could show the existence of individual LDs, it would be of evidence but better be coroborated by other methodology. In this context it should also be note that LD function as well as biogenesis might possess cell-type specific differences. It could very well be that a hepatocyte which is very active in lipid metabolism possess a highly regulated machinery for LD biogenesis and turnover, while a fibroblast may only contain rudiments of this machinery and in consequence is less well organized with respect to LD organization.

A second question that is of general importance concerns LD biogenesis as well as the organelle lipid metabolism. If one assume that LD are organelles well separated from the ER, can they still incorporate triacylglycerol and/or can they still import precursors for triacylglycerol synthesis? If not, this would mean that only when still in connection with the ER fatty acids are metabolized to triacylglycerol which gets incorporated into the growing LD. If the answer is yes, this would mean that an individual LD must be able to transport triacylglycerol from the cytosol across its single leaflet membrane into the interior. Similarly a special mechanism has to be present in case triacylglycerol precursors are imported into the LD. In consequence of the last idea LDs would also contain machinery to metabolize such precursors as well as those enzymes to eventually break down the stored LD contents such as triacylglycerol or cholesterol esters. Within this context one has to be aware of the

fact that it is also unclear in which way highly hydrophobic molecules such as triacylglycerol are transported through the cytosol.

In with context my own observation might be of interest. When analyzed by immunofluorescence, up to 4h incubation with oleic acid, the exposed cells contained LDs fully surrounded by TIP47 but devoid of Nile red staining (Figure 4.10., Figure 4.11). The yellow spectral emission of Nile Red is indicative of high hydrophobisity (Greenspan et al., 1985b). In conclusion more mature LDs containing high concentrations of neutral lipid and exhibiting bright staining of Nile red differs from "younger" LDs with respect of their hydrophobisity. One interpretation of this finding could be as follows: when extracellular concentration of free unesterified fatty acids is high, LDs appear to accumulate first substrates or intermediates of triacylglycerol synthesis, which are later converted into TAG. If this is true, the phospholipid monolayer of nascent LDs should be highly destabilized by the contents. This is why LD might need a number of scaffolding/structural proteins and, probably, chaperons to maintain the integrity of their surface. Such proteins may include members of the family of PAT proteins such as TIP47.

5.3 The function of TIP47 in LD biogenesis

We have unequivocally shown that endogenous (Figure 4.3., Figure 4.7) as well as tagged TIP47 (Figure 4.26.) is a constituent of LDs and confirmed that its recruitment to the organelles is induced by administration of fatty acids, in particular oleic acid (Wolins et al., 2001). Later analysis of the time course of recruitment demonstrated that TIP47 decorates nascent LDs rather than mature ones which are indicated by Nile red staining (Figure 4.10.). TIP47 recruitment occurs early upon administration of fatty acids and, under our experimental conditions, achieves its maximum before LDs mature (summarized in Figure 5.1.). We then analysed different aspects of LD biogenesis or processes preceding it with respect to a putative function of TIP47.

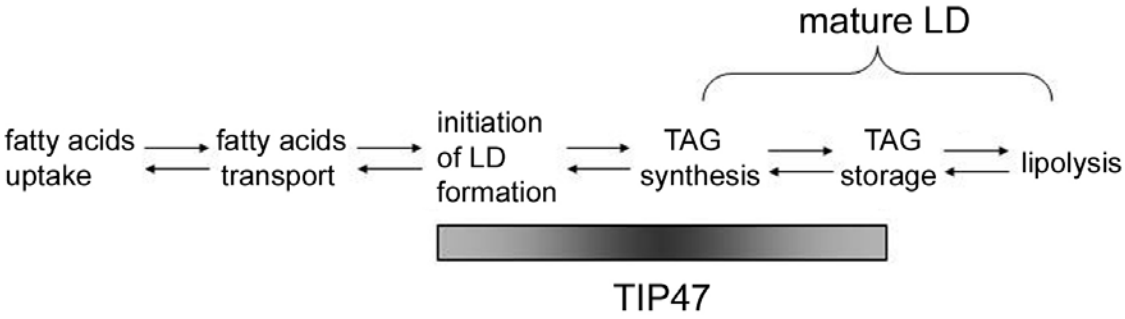

Figure 5.1. Shown is the maximal recruitment of TIP47 (indicated by the colour intensity) within life cycle of LDs.

We found that more than 90% depletion of TIP47 by RNAi does not affect the efficiency of long chain fatty acids uptake by HeLa cells (Figure 4.19.) and has no major effect on both the protein and the phospholipid composition of LDs (Figure **4.21**., Figure 4.22.). However, it significantly impairs the morphology of the LD population (Figure 4.18.) and decreases the amount of fatty acids incorporated into LDs by 50% (Figure 4.20.). We propose that TIP47 may function as a scaffolding protein important to ensue rapid formation of LDs upon starvation (discussed in 4.1.6.) and to maintain the integrity of nascent LD. The suggestion could explain the alterations in TIP47 RNAi cells. The LDs of the affected cells became larger and fewer since the lowered level of TIP47 may coat only some LDs and more fatty acids are transported to the same droplet to compensate for the lack of TIP47 (surface to volume ratio is lower). However, the total amount of stored material still was reduced when cells were incubated in presence of oleic acid for 12h. Under our experimental conditions the remaining small amount of TIP47 might be still sufficient to continuously promote the formation of new LDs, which would explain why the RNAi cells could accumulate roughly normal amounts of lipid after longer periods of exposure. Therefore, the effect of TIP47 suppression is most dramatic early after exposure of cells to fatty acids.

A TIP47 function in stabilization of the phospholipid monolayer of nascent LDs would be of special significance if amphipathic precursors of triacylglycerol, such as fatty acids and diacylglycerol, would, indeed, accumulate in the “young” LDs (see 5.2.). In this scenario, lack of TIP47 would inhibit LD growth and maturation because, e.g.

diacylglycerol, due to its physicochemical properties, would destabilize the ordered phospholipid structures (Goni and Alonso, 1999).

Alternatively, TIP47 may function as a fatty acid transport protein, directly participating in delivery and incorporation of fatty acids into the LDs. The hypothesis is supported by the fact that TIP47 is abundant in the cytoplasm and that its recruitment to LD is efficiently induced by addition of fatty acids. However it is not clear yet whether its association with LD surface is transient and whether the protein is continuously recycling to and from the organelles. Similar proposals were made for adipophilin, the most closely related protein to TIP47. The authors suggested that adipophilin is a saturable fatty acid transport protein (Gao and Serrero, 1999; Serrero et al., 2000) or that it is important for cholesterol targeting to LD (Frolov et al., 2000), nevertheless, the function of adipophilin is not entirely clear yet (see 1.1.3.1.).

It was assumed earlier that TIP47 might play a role in LDs aggregation while it has coiled-coil structures, which could enable its dimerization or even oligomerization (Than et al., 2003), however, it could not explain the phenotype of the TIP47 RNAi cells.

5.4 TIP47 recruitment to the LDs

Proteins studied by now with respect of their association with LDs can be divided into four groups:

1) plant and viral proteins containing long hydrophobic stretches embedded into the phospholipid monolayer and, probably, the core of LDs, and requiring the “proline knot” motif as in oleosins and caleosins (Murphy, 2001). Hepatitis C virus (HCV) and GB virus-B core proteins (Hope et al., 2002) are examples;
2) proteins containing both long hydrophobic and palmitoylated sequences (caveolins) (van Deurs et al., 2003);
3) proteins recruited to LD surface via a docking protein (e.g. hormone sensitive lipase (Syu and Saltiel, 1999));
4) proteins containing neither long hydrophobic sequences nor known motifs, but demanding long targeting regions with a certain degree of redundancy and, probably, intact secondary structure (perilipin A (Garcia et al., 2003) and

adipophilin (McManaman et al., 2003; Nakamura and Fujimoto, 2003; Targett-Adams et al., 2003)).

The knowledge of the mechanisms of TIP47 recruitment to LDs as well as involved targeting determinants may give us an opportunity to better understand the function of the protein on LD. We could show that the N-terminal half of TIP47 is sufficient for LD targeting (Figure 4.26.).

In this context it is interesting to note that the interaction of perilipins, adipophilin and TIP47 with the LD surface was found to be hydrophobic, none of the proteins can be dislodged from the membranes by alkali carbonate (Londos et al., 1999; Wolins et al., 2001).

Furthermore, the distribution of our TIP47 deletion variants is remarkably similar to that observed for adipophilin mutants, suggesting that a conserved sequence is required. large deletions of either C- or N-termini of TIP47 yield proteins with intracellular distribution similar to that of human adipophilin truncation variants, e.g. HA-TIP47$_{1-239}$ (on LD surface in all transfected cells) and HA-TIP47$_{240-434}$ (diffuse in 100% transfected cells) (Figure 4.26.) have nearly the same distribution as GFP-ADRP$_{1-221}$ (mainly in association with LDs) and GFP-ADRP$_{221-437}$ (diffuse in most of transfected cells), respectively (Targett-Adams et al., 2003) despite the difference in the tags introduced and other experimental conditions.

The mode of interaction of the PAT proteins with LDs is not entirely clear, however, it is worth to mention several proposals made.

In one of the early publications, it was suggested that human adipophilin could be anchored to membranes via covalently bound lipid moieties, while there were some evidence that the protein is acylated between residues 93-109 with 5-6 mol of acyl groups per 1 mol of protein (Heid et al., 1996). But the proposal seems unlikely, since others could show that the region of the protein is not required for association with LDs (Nakamura and Fujimoto, 2003; Targett-Adams et al., 2003) (Figure 5.2).

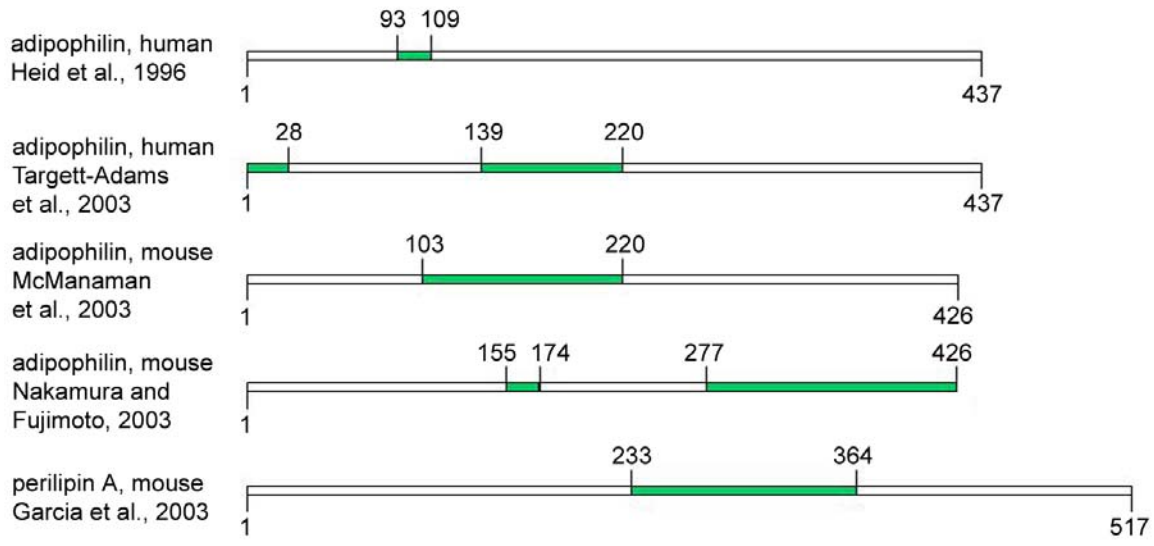

Figure 5.2. LD targeting regions of adipophilin and perilipin A. Regions found to be most important for LD targeting are shown in green.

Lu et al. (Lu et al., 2001) hypothesized that the N-terminal high homology PAT domain is essential for function and/or subcellular localization, perhaps as a targeting moiety to the surface of LD. However, till now no proof for importance of the PAT domain or its common function in the protein family was obtained. Remarkably, the PAT domain turned out to be of no special significance for LD targeting of different PAT family members: human (Heid et al., 1996; Targett-Adams et al., 2003) or mouse (McManaman et al., 2003; Nakamura and Fujimoto, 2003) adipophilin, perilipin A (Garcia et al., 2003) and it is not sufficient for LD association of TIP47 according to our data (Figure 4.26).

Most recently it was suggested that the key feature which determines the interaction of adipophilin with LDs are amphipathic α-helices (McManaman et al., 2003) found also in apolipoproteins (Segrest et al., 1992). Furthermore, it would be interesting to investigate whether TIP47 and other PAT proteins behave similar to α-synuclein, the Parkinson's disease protein which is also able to associate with LDs (Cole et al., 2002). α-synuclein is unfolded in solution (Weinreb et al., 1996) but adopt a structure with strong α-helical character in the presence of membranes containing acidic phospholipids (Davidson et al., 1998; Jo et al., 2000). Interestingly, α-synuclein binding to membranes can be stabilized by cross-linking, which results in isolation of dimers and trimers (Cole et al., 2002).

In conclusion further work is required to characterized the exact sequence(s)/motif that target PAT family proteins to LDs. In addition it awaits further poof whether the association with LDs also requires the help of a certain post-translational modification.

6 Future directions

The work performed here is the basis for the future investigation of TIP47 function and early stages of LD biogenesis. We are going

to analyse early stages of LD formation using fluorescently labeled fatty acids and cholesterol as well as GFP-tagged TIP47;

to test whether TIP47 is directly involved in incorporation of fatty acids into LDs and whether its function on LDs is tissue specific;

to search for putative TIP47 partners using various chemical cross-linkers;

to check whether TIP47 phosphorylation/dephosphorylation cycles regulate recruitment of the protein to LDs;

to further analyse LD targeting determinants of TIP47;

to create a single adipophilin and a double adipophilin/TIP47 RNAi cell lines to get an insite into ADRP function and to elucidate whether adipophilin could compensate for lack of TIP47 and to study the effect of RNAi of TIP47 and/or adipophilin in specialized cell types such as adipocytes.

7 Literature

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1982 – 1988	Secondary education in Rjazan, Russia.
1988 - 1992	Secondary education in Tashkent, Uzbekistan.
1993 -1997	Bachelor of Science in Biology, at the Department of Cytology and Histology, St.-Petersburg State University, Russia.
1997-1999	Master of Science in Biology at the Department of Embryology, Diploma with Honors, St.-Petersburg State University, Russia
M.Sc Project	"The interaction of a new hematopoietic progenitor cells marker Ac133 and retinoblastoma gene product". At Laboratory of Regulation of Gene Expression, Institute of Cytology of Russian Academy of Sciences, St.-Petersburg, Russia.
Jan.-Mai 2000	Worked as Project Assistant with Dr. Popov B.V, Institute of Cytology of Russian Academy of Sciences, St.-Petersburg, Russia.
June 2000 - present	Joined the “Zentrum für Biochemie und Molekulare Zell Biologie” (Biochemie II) to pursue doctoral studies under the supervision of Dr. Stefan Höning to work on the research project “TIP47 recruitment to lipid droplets and function on the organelle biogenesis”