Posttranslational modifications of Ancient Ubiquitous Protein 1 control intracellular Lipid Droplet clustering

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Daniel Lohmann
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1. Gutachter: Prof. Dr. Christoph Thiele
2. Gutachter: Prof. Dr. Thorsten Lang

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SUMMARY

Lipid droplets (LDs) are the intracellular storage organelles for neutral lipids. Over the last decade LDs have been identified to play important roles in lipid homeostasis, cellular signaling events and as a platform to sequester specific proteins. Lipid droplets are also linked to human diseases like type 2 diabetes or obesity. LDs are dynamic organelles, which can move rapidly within cells along microtubule tracks using the cellular transport machinery. While LDs are usually found evenly dispersed throughout the cytoplasm of cells, they have also been observed to aggregate and form dense clusters consisting of numerous individual LDs. It has been suggested that LD clustering is involved in LD fusion or in mediating increased storage of neutral lipids. Even though several proteins have been shown to induce LD clustering, a molecular mechanism explaining how LDs are tethered to form these clusters is unknown.

This thesis demonstrates that LD associated ancient ubiquitous protein 1 (AUP1) promotes the clustering of LDs. Furthermore, it is shown that knock-down of AUP1 leads to the dispersion of LD clusters. Results from this study demonstrate that AUP1 is ubiquitinated in a process that depends on the integrity of an intrinsic AUP1 ubiquitin-binding domain. It is also shown that AUP1 is ubiquitinated at several lysine residues. AUP1 mutants that fail to become ubiquitinated lose the ability to promote LD clustering. Fusing a single ubiquitin moiety to AUP1 mutants that otherwise fail to induce LD clustering, is sufficient to restore LD clustering in cells. Thus, for the first time a posttranslational modification is identified that controls the clustering of LDs. In addition, AUP1 is known to be a highly phosphorylated protein and this study shows that mimicking phosphorylation of one specific threonine residue attenuates AUP1-induced LD clustering. It further shows that this phosphorylation of AUP1 does not interfere with the ubiquitination of AUP1.

Based on these findings, a regulatory mechanism is proposed in which the identified posttranslational modifications of AUP1 control intracellular LD clustering.

ABBREVIATIONS

ACSL3	acyl-CoA synthetase long-chain family member3
ADRP	adipose differentiation-related protein
AUP1	ancient ubiquitous protein 1
AT	acyltransferase domain
Amp	ampicillin
BB	blocking buffer
CUE	coupling of ubiquitin conjugation to ERAD
CIDEA	cell death-inducing DFFA-like effector a
DGAT2	diacylglycerol O-acyltransferase 2
dH_2O	distilled water
DIO2	deiodinase, iodothyronine, type II
DMEM	Dulbecco´s modified Eagles medium
DMSO	dimehtylsulfoxide
ER	endoplasmic reticulum
ERAD	ER associated degradation
Eps15	epidermal growth factor pathway substrate 15
EGFR	epidermal growth factor receptor
E. coli	*Escherichia coli*
FCS	fetal calf serum
fsp27	fat specific protein27
FAS2/UBXD8	fas associated factor family member 2
GRASP	golgi reassembly and stacking protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
G2BR	G2 binding region
His	histidin
HCV	hepatitis C virus
HRP	horseradish peroxidase
HA	hemagglutinin

ABBREVIATIONS

isoT	isopeptidase T
K_d	dissociation constant
Kan	kanamycin
kDa	kilo dalton
LDT	lipid droplet targeting
LD	lipid droplet
LPCAT	lysophosphatidylcholine acyltransferase
LC3	microtubule-associated protein-1 light chain 3
MEM	minimal essential medium Eagel
MettL7b	methyltransferase like 7b
NEDD4	neural precursor cell expressed developmentally down-regulated 4
NSDHL	NAD(P)H steroid dehydrogenase-like protein
PC	phosphatidylcholine
PFA	paraformaldehyde
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
Poli	polymerase iota
PKA	cAMP dependent protein kinase
P62/SQSTM1	ubiquitin binding protein of 62kDa/sequestosome1
RABEX-5	rabaptin-5 associated exchange factor for Rab5
RT	room temperature
RUZ	rabex-5 ubiquitin binding zinc finger
siRNA	small interfering RNA
SDS	sodium dodecyl sulfate
SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
TRAPPI	transport protein particle I
TAG	triacylglycerides
UBL	ubiquitin-like domain
Ub	ubiquitin
UBA	ubiquitin associated
UBM	ubiquitin binding motif

ABBREVIATIONS

Ube2g2	ubiquitin conjugating enzyme E2 G2
UBD	ubiquitin-binding domain
UBX	ubiquitin regulatory X
WT	wild type
WB	western blot
ZFN	zinc finger nuclease
ZIP	zinc transporter
ZnF UBP	zinc-finger ubiquitin binding

1. Introduction

1.1. Lipid Droplets

Lipid Droplets (LDs) are neutral lipid storage organelles found in most organisms, from bacteria and yeast to plants and mammals (Zweytick *et al.*, 2000). LDs consist of a hydrophobic core, mainly made up of triacylglycerides (TAG) and esterified sterols, surrounded by a phospholipid monolayer (Fig. 1). Compartmentalization via a polar lipid monolayer is a unique structural feature which LDs share only with plasma lipoproteins (Walther and Farese, 2012). First observed in the 19th century by light microscopy (R., 1890; Debelyy *et al.*, 2011), LDs have long been regarded as rather inert lipid inclusions with a sole purpose of storing fat. However, research on LDs over the last decades revealed LDs as independent cellular organelles with distinct functions apart from the storage of lipids. LDs have been shown to play an important role in lipid homeostasis (Thiele and Spandl, 2008; Gong *et al.*, 2009), signaling events (Bozza *et al.*, 2011) and are thought to function as a platform to sequester excess proteins or otherwise toxic substances (Ohsaki *et al.*, 2006; Welte, 2007; Cermelli *et al.*, 2006). LDs are linked to several human diseases like obesity or type 2 diabetes (Greenberg *et al.*, 2011). In addition, human pathogens like the hepatitis C virus (HCV) or *Chlamydia trachomatis* can hijack LDs as sites for assembly or replication (Shavinskaya *et al.*, 2007; Kumar *et al.*, 2006).

LDs themselves are adorned by proteins and several LD proteomes revealed a plethora of different proteins associated with LDs (Athenstaedt *et al.*, 1999; Brasaemle *et al.*, 2004; Fujimoto *et al.*, 2004; Wan *et al.*, 2007). In 1991, perilipin was the first protein to be identified to specifically associate with LDs in vertebrates (Greenberg *et al.*, 1991). Within the last 20 years several LD proteins were identified and described, giving new insights into LD formation, growth, function and metabolism (Beller *et al.*, 2010; Martin and Parton, 2006; Murphy *et al.*, 2009; Thiele and Spandl, 2008).

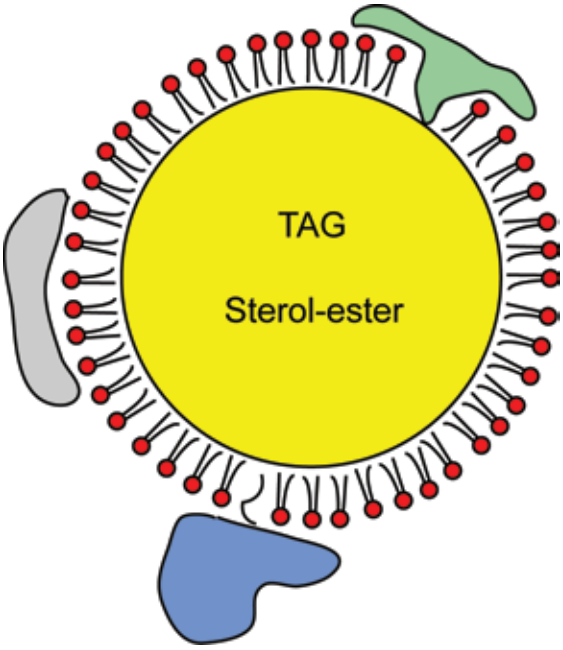

Figure 1.

Schematic view of an LD

Schematic representation of an LD is shown. A hydrophobic core mainly composed of TAG and esterified sterols is surrounded by a phospholipid monolayer with several associated and embedded proteins. Picture kindly provided by Mario Schöne.

How and where LDs form is still under debate. Many cellular organelles self-replicate by fission, however LDs are thought to form *de novo*. A current but yet unproven model for LD formation is shown in Figure 2. Neutral lipids accumulate between the two ER leaflets until a new LD buds from the ER, taking with it the cytosolic membrane sheet from the ER, which in turn becomes the phospholipid monolayer surrounding the neutral lipid core of the LD (Martin and Parton, 2006).

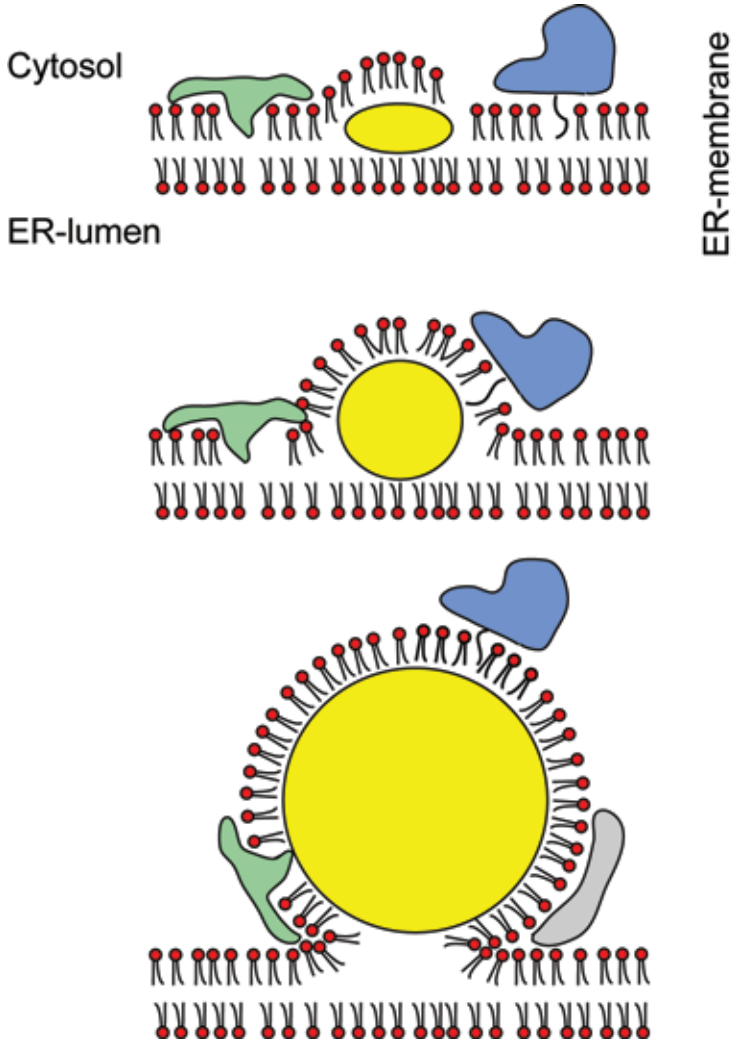

Figure 2.

A current model for the *de novo* formation of an LD

Neutral lipids accumulate between the two ER leaflets which marks the site for the *de novo* formation of an LD. The nascent LD grows by acquiring more neutral lipids. Simultaneously proteins attach to the forming LD which finally buds from the ER. Picture kindly provided by Mario Schöne.

After *de novo* formation, LDs are not static with respect to size, localization or protein composition. TAG can be synthesized locally on the surface of LDs by the activity of diacylglycerol O-acyltransferase 2 (DGAT2) and is thought to be stored within the hydrophobic core of LDs (Kuerschner *et al.*, 2008). Phosphatidylcholine (PC), the main component of the LD phospholipid monolayer, can be synthesized locally by the activity of lysophosphatidylcholine acyltransferase (LPCAT) 1 and 2, which could

provide membrane lipids for growing LDs (Moessinger *et al.*, 2011). Although a rare event, LDs have also been suggested to grow by fusion of individual LDs (Boström *et al.*, 2005; Bostrom *et al.*, 2007; Walther and Farese, 2009) or by shuttling of lipids from one LD to another, requiring both LDs to be in close contact with each other (Gong *et al.*, 2011).

1.2. LD clustering

LDs have a diverse morphological appearance, ranging in size from less one µm in yeast and up to 100 µm in adipocytes (Walther and Farese, 2009; Zweytick *et al.*, 2000). But not only the size of LDs can vary; also their intracellular distribution is modular. LDs have been observed to move rapidly within cells (Targett-Adams *et al.*, 2003; Spandl *et al.*, 2009) and that motility and intracellular redistribution of LDs depends on an intact microtubule network (Bostrom *et al.*, 2005; Boulant *et al.*, 2008; Targett-Adams *et al.*, 2003). It was further shown that LD motility depends on the motor proteins dynein (Gross *et al.*, 2000; Welte *et al.*, 2005; Yu *et al.*, 2011) and kinesin-1 (Shubeita *et al.*, 2008). LD motility has been demonstrated to be important for the change in LD distribution in early *Drosophila* embryogenesis (Gross *et al.*, 2003; Welte *et al.*, 1998; Yu *et al.*, 2011). LD motility has also been suggested to be important for the exchange of lipids between LDs and distinct cellular compartments (Zehmer *et al.*, 2009b; Bartz *et al.*, 2007a; Liu *et al.*, 2007; Ozeki *et al.*, 2005; Goodman, 2008). The microtubule network and the motor protein dynein have also been suggested to be necessary for the clustering of LDs (Boström *et al.*, 2005; Welte, 2009; Boulant *et al.*, 2008; Goodman, 2008).

Even though LDs are usually dispersed throughout the cytosol (Welte, 2009; Walther and Farese, 2012), under certain conditions LDs have been observed to aggregate and form densely packed clusters, consisting of numerous individual LDs (Fowler and Greenspan, 1985; Walther and Farese, 2012; Scott *et al.*, 2004). This LD clustering has been suggested to be a prerequisite for LD fusion (Jambunathan *et al.*, 2011; Gong *et al.*, 2011) whereas the opposite effect, LD declustering is thought to promote increased access to LDs by cytosolic lipases, thereby facilitating lipolysis

(Marcinkiewicz *et al.*, 2006; Garcia *et al.*, 2004; Christianson *et al.*, 2010; Bickel *et al.*, 2009). Also, core protein of HCV localizes to LDs and causes their clustering and it has been suggested that this redistribution of LDs increases pathogenicity of HCV (Boulant *et al.*, 2008; Depla *et al.*, 2010; Herker and Ott, 2011; Shavinskaya *et al.*, 2007).

Sample images from two cell lines used in this work and depicting the different intracellular distributions of LDs are shown in Figure 3. COS-7 cells supplemented with oleate exhibit numerous LDs dispersed throughout the cell (Fig. 3, A) whereas A431 cells exhibit few clusters of numerous individual LDs (Fig. 3, B).

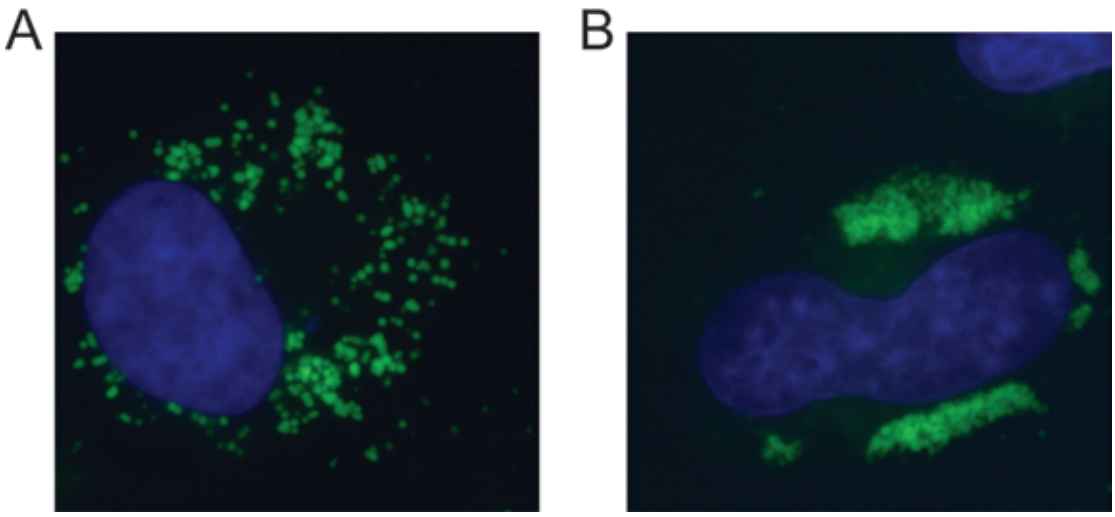

Figure 3.

LD distribution in COS-7 and A431 cells

Fluorescent micrographs of two different cell-lines supplemented with 50 µM oleate are shown with LDs depicted in green and nuclei in blue. (A) COS-7 cells exhibit numerous LDs dispersed throughout the cytosol with no apparent clustering of LDs. (B) A431 cells show few densely packed LD clusters consisting of numerous individual LDs.

Several proteins have been shown to promote LD clustering upon overexpression in cells but a molecular mechanism explaining how individual LDs are tethered to form LD clusters is currently unknown. LD proteomics revealed several proteins associated with LDs that are commonly known for vesicle mobility and docking to target membranes, mainly proteins of the Rab family (Bartz *et al.*, 2007b; Brasaemle *et al.*, 2004). In addition, several members of the SNARE (soluble N-ethylmaleimide-

sensitive-factor attachment receptor) membrane fusion machinery have been identified on LDs (Bostrom *et al.*, 2007). Rab proteins have been shown to mediate movement of vesicles along the cytoskeleton and suggested to recruit proteins necessary for docking and tethering of vesicles to target membranes (Jordens *et al.*, 2005), whereas SNAREs are important for vesicle fusion events (Jahn and Scheller, 2006). Rab and SNARE proteins might play similar roles in the cellular distribution, clustering and/or fusion of LDs (Bostrom *et al.*, 2007).

Ancient ubiquitous protein 1 (AUP1) is one protein that has been observed to promote LD clustering upon overexpression in COS-7 cells (Spandl Johanna, PhD thesis 2009). Preliminary data also suggest that knock-down of AUP1 in the A431 cell line leads to a partial declustering of the otherwise predominant LD clusters observed in this cell line (Spandl Johanna, PhD thesis 2009). This thesis focuses on the elucidation of a possible molecular mechanism behind AUP1-induced LD clustering.

1.3. Ancient Ubiquitous Protein 1

AUP1 is a ubiquitously expressed and highly conserved protein among multi-cellular organisms that has a predicted length of 410 amino acids (Jang *et al.*, 1996; Spandl *et al.*, 2011). The AUP1 gene was first described as part of mouse chromosome 6 and human chromosome 2 (Jang *et al.*, 1996; Weber *et al.*, 1998) and AUP1 was later described as a cytosolic protein functioning in the signal transduction in platelets (Kato *et al.*, 2002). Yet, AUP1 appeared in several LD proteomes generated from different labs (Brasaemle *et al.*, 2004; Moessinger *et al.*, 2011; Sato *et al.*, 2006; Wan *et al.*, 2007). Immunostaining and subcellular fractionation revealed AUP1 as an LD and ER membrane associated protein (Spandl *et al.*, 2011; Klemm *et al.*, 2011).

AUP1 exhibits a hydrophobic stretch of amino acids close to its N-terminus (henceforward termed lipid droplet targeting (LDT) domain), which is assumed to be necessary for AUP1 localization to both, LDs and ER membranes (Stevanovic and Thiele, 2012; Spandl *et al.*, 2011). Regardless of AUP1 localization to LDs or ER membranes, both the C- and N-termini of AUP1 face the cytoplasm (Fig. 4) (Spandl *et al.*, 2011). Therefore, AUP1 belongs to the category of monotopic membrane

proteins. Indeed, monotopic integration of proteins into the monolayer of LDs and the ER bilayer has been shown for several LD proteins like LPCAT1 and 2 (Moessinger *et al.*, 2011), DGAT2 (Stone *et al.*, 2006) or NAD(P)H steroid dehydrogenase-like protein (NSDHL) (Ohashi *et al.*, 2003; Caldas and Herman, 2003).

Adjacent to the LDT domain resides a predicted acyltransferase (AT) domain, which appears to have weak acyltransferase activity (Spandl Johanna, PhD thesis 2009).

At its C-terminus, AUP1 has two functional domains, a G2 binding region (G2BR) and a coupling of ubiquitin conjugation to ERAD (CUE) domain. The G2BR domain is necessary and sufficient to bind the ubiquitin conjugating enzyme E2 G2 (Ube2g2) and recruit Ube2g2 to LDs (Fig. 4) (Spandl *et al.*, 2011; Klemm *et al.*, 2011). The CUE domain belongs to a larger group of protein domains that have been shown to bind ubiquitin (further outlined below) (Fig. 4). The CUE domain of AUP1 has been suggested to bind ubiquitin and it has been demonstrated that the CUE domain is important for intramolecular ubiquitination of AUP1 itself (Klemm *et al.*, 2011), yet no function could be assigned to this posttranslational modification. However, preliminary data suggests that deletion of the AUP1 G2BR and CUE domain inhibits intramolecular ubiquitination of AUP1 and negatively influences the ability of AUP1 to promote LD clustering (Spandl Johanna, PhD thesis 2009).

Phosphoproteome profiles from different cell lines found AUP1 to be highly phosphorylated at several threonine and serine residues (Dephoure *et al.*, 2008; Kim *et al.*, 2006; Yang *et al.*, 2006). Preliminary data suggested that a specific phosphorylation of AUP1 at threonine 68 negatively interferes with the ability of AUP1 to induce LD clustering (Spandl Johanna, PhD thesis 2009). Interestingly, a recent study suggested that phosphorylation of the LD associated protein perilipin A leads to the declustering of LD clusters (Marcinkiewicz *et al.*, 2006).

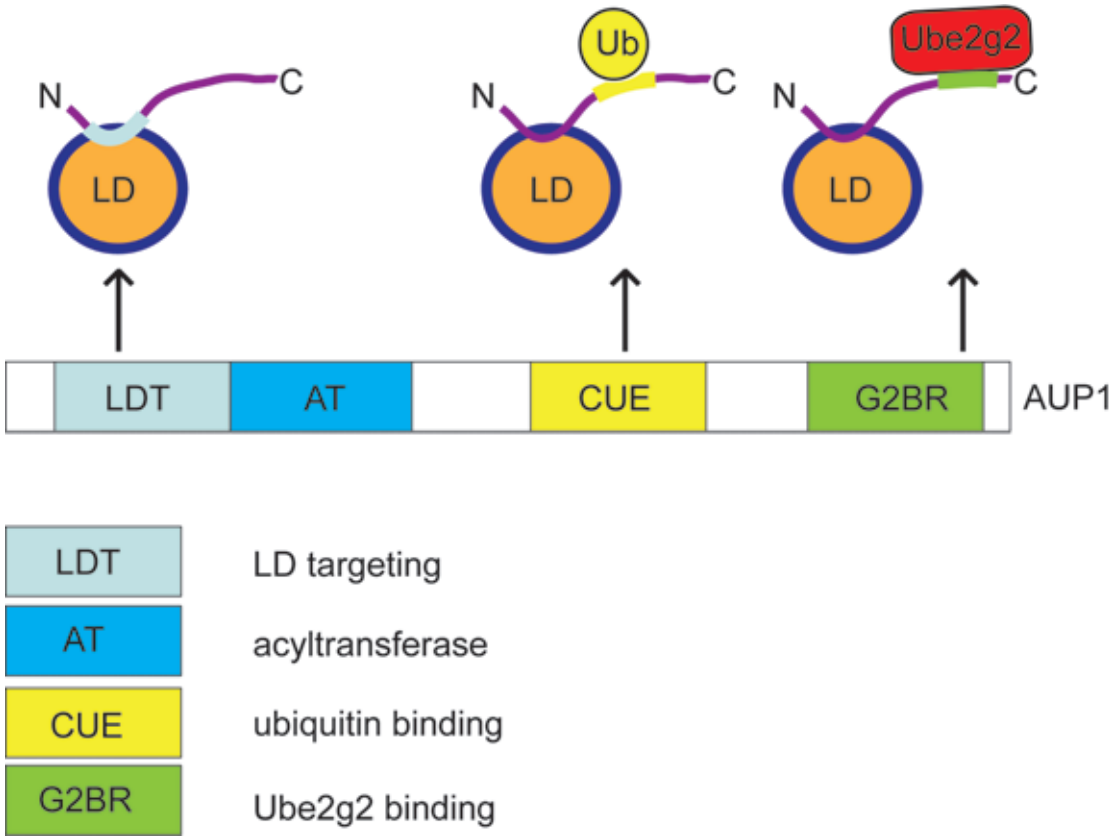

Figure 4.

AUP1 domain structure

Schematic representation of the AUP1 domain structure and their proposed functions are shown. The LDT domain at the N-terminus is important for targeting of AUP1 to LDs and is followed by the predicted AT domain. There are two functional domains at the C-terminus. The CUE domain is important for intramolecular ubiquitination of AUP1 and is thought to bind ubiquitin and the G2BR domain binds the E2 ubiquitin conjugase Ube2g2.

A phyllogenetic analysis of AUP1 uncovered a close correlation to Cue1p in yeast and to the E3 ubiquitin ligase autocrine motility factor receptor (AMFR) in higher eukaryotes (Spandl *et al.*, 2011). All three proteins share the conserved CUE and G2BR domains (Spandl *et al.*, 2011). Cue1p and AMFR participate in ER associated degradation (ERAD), in which misfolded or unassembled proteins from the ER are targeted for destruction by the proteasome (Meusser *et al.*, 2005; Vembar and Brodsky, 2008). AUP1 has been repeatedly suggested to participate in ERAD by recruiting already ubiquitinated ER proteins, binding components of ER quality control machinery or by directing Ube2g2 to sites of protein ubiquitination at the ER

(Claessen *et al.*, 2010; Klemm *et al.*, 2011; Mueller *et al.*, 2008; Spandl *et al.*, 2011). However, the specific role of AUP1 in ERAD is still unknown.

1.4. Ubiquitin

Ubiquitin is a 76 amino acid long globular protein that is found in all eukaryotic life forms (Kerscher *et al.*, 2006). Ubiquitin itself can be covalently attached to other proteins in a highly specific and regulated process, termed ubiquitination. The best understood function for protein ubiquitination is in targeting proteins for degradation by the proteasome, but ubiquitination has additional functions in such diverse processes as endocytosis, histone modification or membrane transport (Weake and Workman, 2008; Schnell and Hicke, 2003; Chen and Sun, 2009). The degradation of proteins by the ubiquitin-proteasome system consists of two main events. First, through the consecutive action of three classes of enzymes a polyubiquitin chain is assembled on the target protein and second, the polyubiquitinated protein is targeted to and degraded by the proteasome (Fig. 5) (Hershko and Ciechanover, 1998). Ubiquitin is commonly attached to the target protein by the formation of an isopeptide bond between the C-terminal glycine of ubiquitin and a lysine residue within the target protein (Hershko and Ciechanover, 1998).

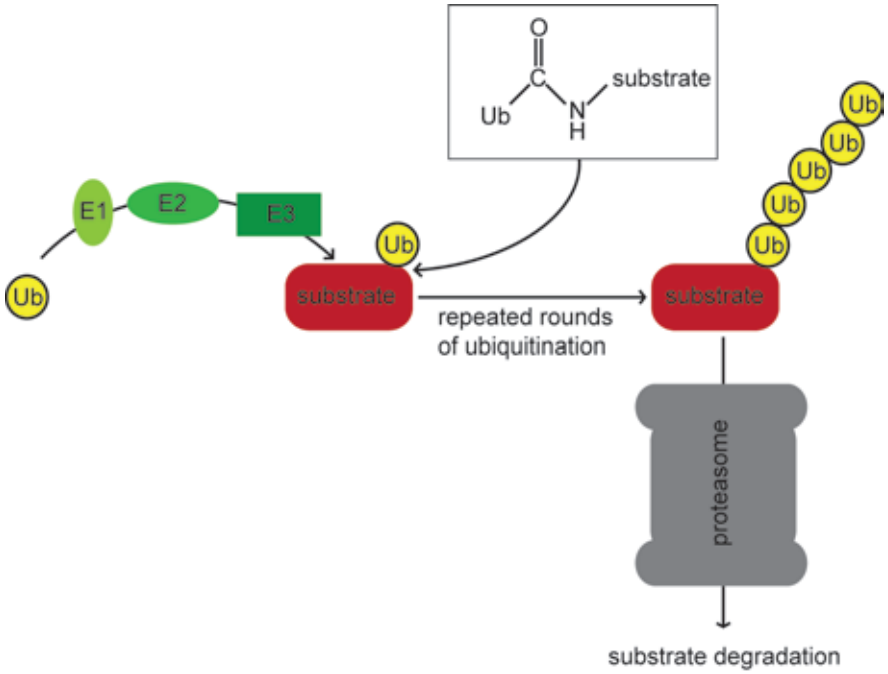

Figure 5.

The ubiquitin-proteasome system

Ubiquitin is attached to a specific substrate by the consecutive action of E1 (or ubiquitin activating enzyme), E2 (or ubiquitin conjugating enzyme), and E3 (or ubiquitin ligase). The C-terminal glycine residue of ubiquitin is attached to a lysine residue within the target protein through the formation of an isopeptide bond. Additional ubiquitin moieties are transferred to the already attached ubiquitin until a polyubiquitin chain is assembled on the substrate, which is subsequently degraded by the proteasome.

For polyubiquitin chain assembly, ubiquitin moieties are covalently linked to each other through isopeptide bonds between a lysine residue of one moiety already attached to the target protein and the C-terminal glycine of the next moiety (Fang and Weissman, 2004). Proteasomal degradation of proteins is mainly mediated by polyubiquitin chains linked through lysine 48 (Hershko and Ciechanover, 1998). However, ubiquitin chains linked through different lysine residues on ubiquitin have been shown to be important for proteasomal degradation as well (Ikeda and Dikic, 2008; Jin *et al.*, 2008; Saeki *et al.*, 2009).

1.5. Monoubiquitination

Ubiquitin attached to a protein is not always further ubiquitinated to form a polyubiquitin chain; proteins can be modified by a single ubiquitin moiety, a process termed monoubiquitination (Johnson, 2002). Protein monoubiquitination, like polyubiquitination, is a highly regulated protein modification, which controls a diverse range of cellular processes (Fig. 6) (Schnell and Hicke, 2003). Monoubiquitination of cell surface receptors can lead to their endocytosis and cycling to lysosomes (Haglund *et al.*, 2003; Sigismund *et al.*, 2005). Monoubiquitination of histones exerts transcriptional control over many genes (Zhou *et al.*, 2008; Weake and Workman, 2008). Monoubiquitination of proteins can also control their subcellular localization, conformation or interaction with other proteins (Haglund and Dikic, 2005).

It has been shown that proteins can be monoubiquitinated at several single lysine residues within the same protein. This multiple monoubiquitination of proteins has been shown to be important for endocytotic events in cells (Fig. 6) (Haglund and Dikic, 2005).

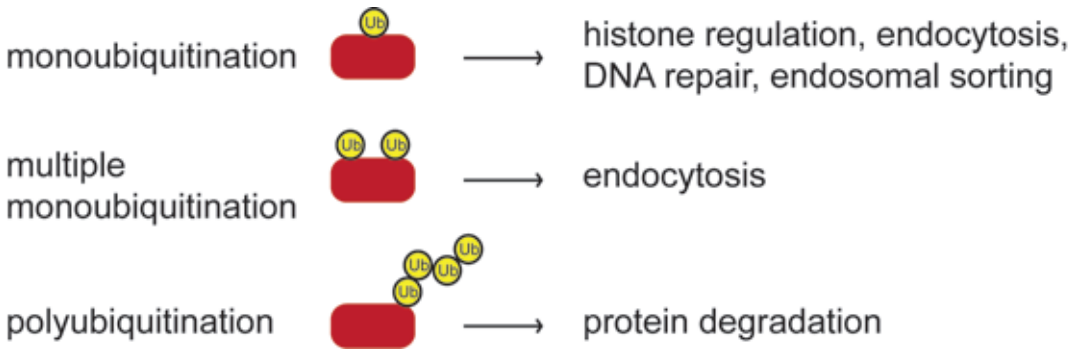

Figure 6.

Protein ubiquitination

Schematic view of different protein ubiquitination variants is shown. A protein can be modified by one ubiquitin moiety (monoubiquitination), an important signal for histone regulation, endocytosis, DNA repair or endosomal sorting. Several individual ubiquitin moieties can be attached to the same protein (multiple monoubiquitination), an important signal for endocytosis. The polyubiquitination of a protein through lysine 48 linked ubiquitin moieties is the major signal for protein degradation by the proteasome.

1.6. Ubiquitin-binding domains

How monoubiquitination of proteins control all these different cellular aspects is as yet not entirely understood. One emerging scheme suggests that a diverse range of effector proteins can interact in a non-covalent fashion with ubiquitin covalently attached to another protein, thereby acting downstream of protein monoubiquitination and translating this modification into its many cellular functions (Fig. 7, A) (Haglund *et al.*, 2003; Haglund and Dikic, 2005; Harper and Schulman, 2006; Ramanathan and Ye, 2012). At least 20 different protein domains, which specifically bind ubiquitin in a non-covalent fashion have been identified so far and have been summarized as ubiquitin-binding domains (UBDs) (Chen and Sun, 2009; Hurley *et al.*, 2006). Proteins containing a UBD are commonly referred to as ubiquitin-receptors. A well-studied ubiquitin-receptor is the epidermal growth factor pathway substrate 15 (Eps15). Eps15 is thought to bind ubiquitin covalently attached to epidermal growth factor receptor (EGFR) by virtue of its UBD and thereby function in EGFR endocytosis (de Melker *et al.*, 2004; van Bergen En Henegouwen, 2009).

In addition, it has been demonstrated that Eps15 itself undergoes monoubiquitination (Klapisz *et al.*, 2002; Woelk *et al.*, 2006; Hoeller *et al.*, 2006). It has been speculated that this monoubiquitination of Eps15 could serve as a self-inhibitory signal, preventing Eps15 from binding to other ubiquitinated proteins (B). It is thought that the UBD of Eps15 can directly bind to the intramolecular attached ubiquitin, which in turn prevents the UBD from interacting with other ubiquitinated proteins, like EGFR (for a representative model see Fig. 7, C) (de Melker *et al.*, 2004; van Bergen En Henegouwen, 2009).

The monoubiquitination of ubiquitin-receptors, like Eps15, is a feature shared by several UBD containing proteins (Klapisz *et al.*, 2002; Hicke *et al.*, 2005b; Di Fiore *et al.*, 2003) including AUP1 (Klemm *et al.*, 2011). A functional UBD is thought to be a prerequisite for the monoubiquitination of ubiquitin-receptors and hence has been termed coupled monoubiquitination (Fig. 7, B). Coupled monoubiquitination has been worked out in molecular detail for Eps15. I was shown that the UBD of Eps15 binds ubiquitin covalently attached to the E3 ubiquitin ligase NEDD4 (neural precursor cell expressed developmentally down-regulated 4) which in turn transfers an activated

ubiquitin to Eps15 itself (Woelk *et al.*, 2006). Coupled monoubiquitination of Eps15 is also accomplished in an analogous mechanism by the E3 ubiquitin ligase Parkin (Fallon *et al.*, 2006b; Haglund and Stenmark, 2006). Parkin contains a ubiquitin-like (UBL) domain which has been shown to exhibit similar overall structure and fold characteristics to ubiquitin (Chen *et al.*, 2011; Welchman *et al.*, 2005). It has therefore been suggested that the UBL domain of Parkin interacts with the UBD of Eps15 and subsequently transfers a ubiquitin to Eps15 itself (Fallon *et al.*, 2006a; Haglund and Stenmark, 2006).

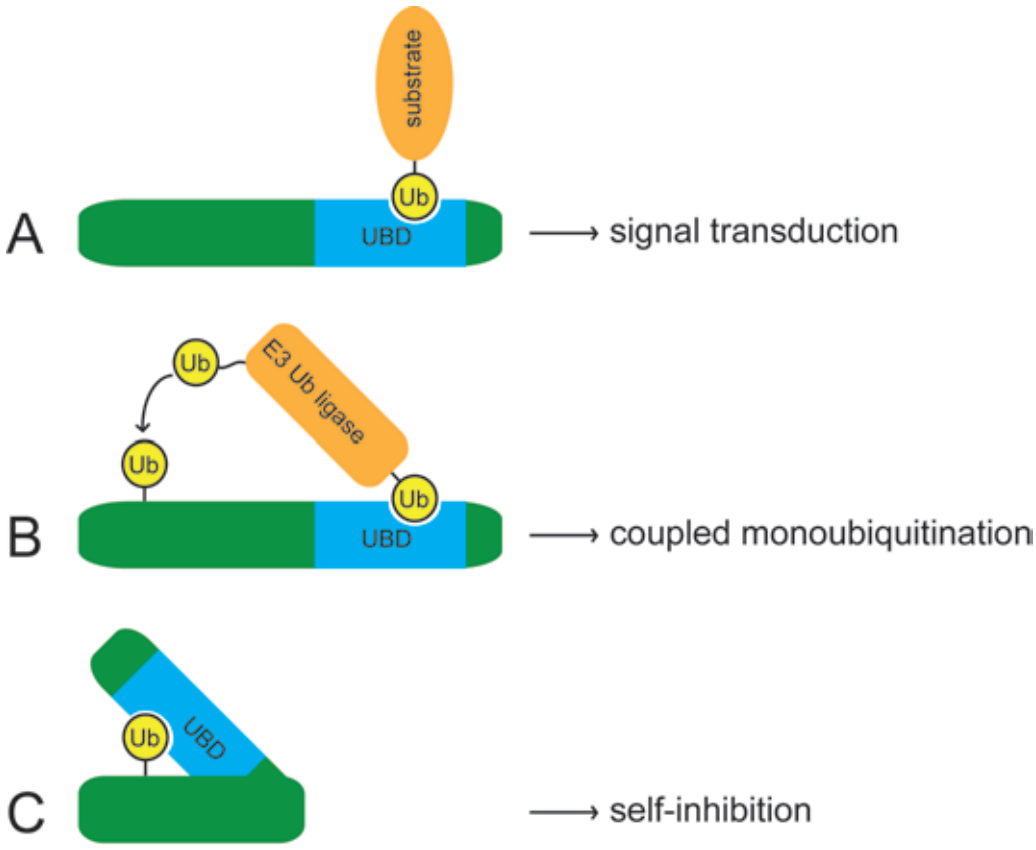

Figure 7.

A ubiquitin-receptor and its diverse interactions with ubiquitin

Schematic representation of a ubiquitin-receptor and its interaction with ubiquitin is shown. (A) UBD containing proteins bind to monoubiquitinated proteins and mediate downstream signaling events. (B) Coupled monoubiquitination leads to the intramolecular monoubiquitination of a ubiquitin-receptor. Ubiquitin covalently attached to an E3 ubiquitin ligase binds to the UBD and thereafter transfers an activated ubiquitin to the UBD containing protein. (C) Intramolecular interaction between a covalently attached ubiquitin and the UBD on the same protein leads to self-inhibition of a ubiquitin-receptor.

1.7. Molecular interaction of ubiquitin and UBDs

Binding of UBDs to ubiquitin relies on specific amino acid residue contacts between ubiquitin and the UBD. Key hydrophobic amino acids of several UBDs have been demonstrated to contact a highly conserved hydrophobic patch on ubiquitin centered around isoleucine 44 and comprising leucine 8 and valine 70 (Chen and Sun, 2009; Hicke *et al.*, 2005b; Hurley *et al.*, 2006). These hydrophobic contacts are crucial for the vast majority of UBD ubiquitin interactions (Chen and Sun, 2009). However, other amino acid residues on ubiquitin have been shown to be important for binding to UBDs. One such exception is the Rabex-5 ubiquitin binding zinc finger (RUZ) domain of RABEX-5 (Rabaptin-5 associated exchange factor for Rab5), which interacts specifically with aspartic acid 58 on ubiquitin (Penengo *et al.*, 2006; Lee *et al.*, 2006). Considering the highly conserved nature of ubiquitin, additional residues on ubiquitin important for UBD interaction might be unraveled in the future (Raiborg *et al.*, 2006; Hicke *et al.*, 2005b). An overview of amino acid residues on ubiquitin mentioned in this thesis is given in Figure 8.

Figure 8.

Several important amino acid residues on ubiquitin

Schematic representation of a ubiquitin moiety with annotated amino acid residues (structure and relative distribution of amino acid residues derived from (Chung *et al.*, 2010; Hicke *et al.*, 2005b; Holstein *et al.*, 2012). A hydrophobic patch consisting of leucine 8 (L8), isoleucine 44 (I44) and valine 70 (V70) is important for binding to most UBDs. Aspartic acid 58 (D58) is important for binding to the RUZ domain of Rabex-5. Glycine 76 (G76) is covalently linked to lysine residues of other proteins or other ubiquitin moieties. Lysine 48 (K48) is the primary residue for covalent attachment of additional ubiquitin moieties to form a polyubiquitin chain.

2. Aim of this thesis

In general, the aim of this thesis is to contribute to the molecular understanding of the cell biology of lipid droplets. Ancient ubiquitous protein 1 (AUP1) is a lipid droplet (LD) associated protein, which has been observed to influence the intracellular distribution of LDs. This thesis intends to characterize and quantify the impact of AUP1 overexpression and knock-down on the intracellular distribution of LDs.
The main body of experiments engages in the elucidation of molecular mechanisms behind AUP1-induced redistribution of LDs. Special focus is laid on the suggested posttranslational modifications of AUP1 through ubiquitination and phosphorylation. The following questions were approached during the work of this thesis:

- Are any of the functional domains of AUP1 necessary for the redistribution of intracellular LDs?
- Is the localization of AUP1 to LDs necessary for its impact on the redistribution of LDs?
- What are the molecular mechanisms behind the ubiquitination of AUP1?
- Which lysine residues of AUP1 are targeted by ubiquitination?
- Does the ubiquitination of AUP1 influence the intracellular distribution of LDs?
- Does the phosphorylation of AUP1 influence the intracellular distribution of LDs?

3. Materials and Methods

3.1. Materials

3.1.1. Chemicals and cell culture media

Cell culture media (DMEM, FCS, trypsin/EDTA, Opti-MEM) were obtained from Life Technologies. DNA preparation was done using NucleoBond Xtra Midi, 740410 and NucleoSpin Plasmid, 740588 from Macherey-Nagel (Düren, Germany) or QIAquick gel extraction kit, 28704 from Qiagen (Hilden, Germany). Chemicals were obtained from AppliChem (Darmstadt, Germany) or Carl Roth (Karlsruhe, Germany). Enzymes (Restriction enzymes, Phosphatases, DNA-Polymerase (Phusion High Fidelity), dNTPs, loading dye, protein and DNA ladders were obtained from New England BioLabs (Ipswich, United States). Primers were obtained from Biosprings (Frankfurt, Germany).

3.1.2. siRNAs, Constructs, Primers

The following siRNAs targeting AUP1 were obtained from Life Technologies:
siRNA1: AUP1 Stealth Select RNAi siRNA (HSS141340)
siRNA2: AUP1 Stealth Select RNAi siRNA (HSS141341)
siRNA3: AUP1 Stealth Select RNAi siRNA (HSS182853)

Table 1: List of Constructs and Primers

Name	Alias	Vector/ Resistance	Primer and Template or Source	Restriction Sites
AUP1-HA	pCT7	pCDNA3.1Hygro-3HA ampicillin	hAUP1_HA_Hindfor CTAGATAAGCTTGCCATGGAGCTTCCCTCAGGGCC hAUP1_HA_Xba_rev CTATCTAGAGTCAGCCTCCTGGGCTCG Template: AUP1-EST	HindIII/ XbaI
AUP1-ΔG2BR-HA	pCT15	pCDNA3.1Hygro-3HA ampicillin	hAUP1_HA_Hindfor CTAGATAAGCTTGCCATGGAGCTTCCCTCAGGGCC AUP_trunc2_nostop_xba CTATCTAGAGCTGGGAAACTTGGAGGCAG Template: pAUP1	HindIII/ XbaI

AUP1-T68D-HA	pCT116	pCDNA3.1Hygro-3HA ampicillin	Source: pCT92	HindIII/ XbaI
AUP1-T68A-HA	pCT117	pCDNA3.1Hygro-3HA ampicillin	Source: pCT93	HindIII/ XbaI
3HA-Ub	pWZ1	ampicillin	Wolfgang Zachariae	
6His-Ub	pWZ2	ampicillin	Wolfgang Zachariae	
AUP1-mutLDT1-HA	pAS1	pCDNA3.1Hygro-3HA ampicillin	AUP1-P33L-V34L-G35L-HA As described in (Stevanovic and Thiele, 2012)	
AUP1-mutLDT2-HA	pAS2	pCDNA3.1Hygro-3HA ampicillin	AUP1-R62F-R63F-HA As described in (Stevanovic and Thiele, 2012)	
AUP1-mutCUE1-HA	pDL45	pCDNA3.1Hygro-3HA ampicillin	AUP1_CUE_VLP-GGR_for GCTCAGAGAGTCAAGGAAGGTGGGCGCCATGTGCCATTGGG AUP1_CUE_VLP-GGR_rev CCCAATGGCACATGGCGCCCACCTTCCTTGACTCTCTGAGC AUP1_HindIII_for GTGCCAAGCTTGCCATGGAGC AUP1_rev GGATCCTCTAGAGTCAGC Template: pCT7	HindIII/ XbaI
AUP1_CUETI\AD_3HA	pDL64	pCDNA3.1Hygro-3HA ampicillin	AUP1_CUE_TI\AD_for GGCTGTGTAGACTTGGCTGACACTAATCTGCTTGAGG AUP1_CUE_TI\AD_rev CCTCAAGCAGATTAGTGTCAGCCAAGTCTACACAGCC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-mutCUE2-HA	pDL71	pCDNA3.1Hygro-3HA ampicillin	AUP1_CUE_VIQRDL\VRQRVD_for CCATGTGCCATTGGGTGTCCGCCAGAGAGTCGAGGCCAAGACTGGCTGTGTAGAC AUP1_CUE_VIQRDL\VRQRVD_rev GTCTACACAGCCAGTCTTGGCCTCGACTCTCTGGCGGACACCCAATGGCACATGG AUP1_HindIII_for and AUP1_rev Template: pDL64	HindIII/ XbaI
AUP1-K305R-HA	pDL75	pCDNA3.1Hygro-3HA ampicillin	AUP1_K305R_for GCTCAGAGAGTCCGGGAAGTTTTGCC AUP1_K305R_rev GGCAAAACTTCCCGGACTCTCTGAGC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-K322R-HA	pDL76	pCDNA3.1Hygro-3HA ampicillin	AUP1_K322R_for CCAGAGAGACCTGGCCCGGACTGGCTGTGTAGACTTG AUP1_K322R_rev CAAGTCTACACAGCCAGTCTTGGCCAGGTCTCTCTGG AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-K143R-HA	pDL87	pCDNA3.1Hygro-3HA ampicillin	AUP1_K1/R_for GGTGGAGTCACTCAGGAGATTCTGTGCTTCC AUP1_K1/R_rev GGAAGCACAGAATCTCCTGAGTGACTCCACC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-K250R-HA	pDL89	pCDNA3.1Hygro-3HA ampicillin	AUP1K2Rnew-for CAGCTGGTGGCCAGGGAATTGG AUP1_K2/R_rev CCCTGTCTGGCCCAATTCCCTGGCCACCAGC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-K264R/K269R-HA	pDL91	pCDNA3.1Hygro-3HA ampicillin	AUP1_KK3-4/RR_for CCAGCTGACAGAGCAGAGCACATGAGGCGACAAAGACACCCC AUP1_KK3-4/RR_rev GGGGTGTCTTTGTCGCCTCATGTGCTCTGCTCTGTCAGCTGG AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI

AUP1-K347R-HA	pDL130	pCDNA3.1Hygro-3HA ampicillin	AUP1_K347R_for GCCTGAAGACATCACCCGGGGAACTCAGTCC AUP1_K347R_rev GGACTGAGTTCCCCGGGTGATGTCTTCAGGC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-K359R-HA	pDL131	pCDNA3.1Hygro-3HA ampicillin	AUP1_K359R_for GCCTCTGCCTCCCGGTTTCCCAGCTCTGG AUP1_K359R_rev CCAGAGCTGGGAAACCGGGAGGCAGAGGC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1-mutCUE3-HA	pDL174	pCDNA3.1Hygro-3HA ampicillin	AUP1_L333E_L334D_for CTATCACTAATGAGGATGAGGGGGCCG AUP1_L333E_L334D_rev CGGCCCCCTCATCCTCATTAGTGATAG AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
Ub-K48R-noStop	pDL189	pCDNA3 ampicillin	Ub_K48R_for GCTGGGCGACAGCTGGAAGATGG Ub_K48R_rev CCATCTTCCAGCTGTCGCCCAGC Ub-EcorI-for CGGGAATTCTCCATGCAGATCTTCG Ub-noStop-XbaI-rev GCCTCTAGAACCACCTCTTAG Template: Ub-3HA	EcoRI/ XbaI
AUP1-UbK48R-HA	pDL196	pCDNA3.1Hygro-3HA ampicillin	AUP1_HindIII_for AUP1_nostop_EcorI_rev GGTAGAATTCGTCAGCCTCCTGGGCTCG Template: pCT7 HindIII/EcoRI cloned into pDL189 HindIII/EcoRI cut again HindIII/XbaI cloned into pCDNA3.1Hygro-3HA HindIII/XbaI	HindIII/ XbaI
AUP1-mutCUE2-UbK48R-HA	pDL198	pCDNA3.1Hygro-3HA ampicillin	AUP1_HindIII_for and AUP1_nostop_EcorI_rev Template: pDL71 HindIII/EcoRI cloned into pDL189 HindIII/EcoRI cut again HindIII/XbaI cloned into pCDNA3.1Hygro-3HA HindIII/XbaI	HindIII/ XbaI
$AUP1_{340-410}$	pDL206	pCDNA3 ampicillin	AUP1-dCUE-for GCCGTAGAATTCATGCCTGAAG AUP1_rev Template: pCT7	EcoRI/ XbaI
$AUP1_{1-293}$	pDL207	pCDNA3 ampicillin	AUP1-dCUE-rev GCCAGTTGGAATTCAGGAGAAG AUP1_HindIII_for Template: pCT7	HindIII/ EcoRI
AUP1-3MYC	pDL208	MoMyc9-XhoI-3.1Hygro-9DG2 ampicillin	Source: pCT7	HindIII/ XbaI
AUP1-mutG2BR-MYC	pDL211	MoMyc9-XhoI-3.1Hygro-9DG2 ampicillin	AUP1-A397E-R398G-R400E-F401D-T402A-HA) AUP1_ARRFT/EGEDA_for GAATACGAAGGAAGGGAAGACGCAGAGAGACGAGCCC AUP1_ARRFT/EGEDA_rev GGGCTCGTCTCTCTGCGTCTTCCCTTCCTTCGTATTC AUP1_HindIII_for and AUP1_rev Template: pCT7	HindIII/ XbaI
AUP1_Δ295 339_V294E	pDL212	pCDNA3 ampicillin	pDL206 EcoR/XbaI cloned into pDL207 EcoRI/XbaI	EcoRI/ XbaI
AUP1-ΔCUE-HA	pDL213	pCDNA3.1Hygro-3HA ampicillin	Source: pDL212	HindIII/ XbaI
AUP1-ΔCUE-MYC	pDL214	MoMyc9-XhoI-3.1Hygro-9DG2 ampicillin	Source: pDL213	HindIII/ XbaI
AUP1-ΔCUE-T68D-HA	pDL219	pCDNA3.1Hygro-3HA ampicillin	AUP1-T68D-sense, AUP1-T68D-anti, AUP1_HindIII_for and AUP1_rev Template: pDL213	HindIII/ XbaI
AUP1-ΔCUE-T68A-HA	pDL220	pCDNA3.1Hygro-3HA ampicillin	AUP1-T68A-sense, AUP1-T68A-anti, AUP1_HindIII_for and AUP1_rev Template: pDL213	HindIII/ XbaI

AUP1-ΔG2BR-T68D-HA	pDL223	pCDNA3.1Hygro-3HA ampicillin	AUP1-T68D-sense GCAGATTCGTAGTGCGGGACATGTGTGCGGTGCTAGG AUP1-T68D-anti CCTAGCACCGCACACATGTCCCGCACTACGAATCTGC AUP1_HindIII_for AUP_trunc2_stop_xba CTATCTAGATTAGCTGGGAAACTTGGAGGCAG Template: pCT7	HindIII/ XbaI
AUP1-ΔG2BR-T68A-HA	pDL224	pCDNA3.1Hygro-3HA ampicillin	AUP1-T68A-sense GCAGATTCGTAGTGCGGGCCATGTGTGCGGTGCTAGG AUP1-T68A-anti CCTAGCACCGCACACATGGCCCGCACTACGAATCTGC AUP1_HindIII_for and AUP_trunc2_stop_xba Template: pCT7	HindIII/ XbaI
NSDHL-UbK48R-HA	pDL254	pCDNA3.1Hygro-3HA ampicillin	NSDHLfull-HindIII-for CTTAAGCTTGCCATGGAACCAGCAG NSDHLfull-EcoRI-rev CTCGAATTCCTTGACCCTCCG Template: pCT76 HindIII/EcoRI cloned into pDL189 HindIII/EcoRI cut again HindIII/XbaI cloned into pCDNA3.1Hygro-3HA HindIII/XbaI	HindIII/ XbaI
AUP1-ΔCUE-UbK48R-HA	pDL263	pCDNA3.1Hygro-3HA ampicillin	AUP1_HindIII_for and AUP1_nostop_EcorI_rev Template: pDL213 Sequential digestion with HindIII/EcoRI cloned into pDL189 HindIII/EcoRI cut again HindIII/XbaI cloned into pCDNA3.1Hygro-3HA HindIII/XbaI	HindIII/ XbaI
NSDHL-noStop	pDL288	pCDNA3 ampicillin	NSDHL-EcorI-for CTTGAATTCGCCATGGAACCAGCAG NSDHL-XbaI-rev CTCTCTAGACTTGACCCTCCG Template: pCT76	EcoRI/ XbaI
UbK48R-NSDHL-HA	pDL290	pCDNA3.1Hygro-3HA ampicillin	Ub-NoLys-HindIII-for CTGAAGCTTTCCATGCAGATCTTCG Ub-NoLys-EcorI-rev GCCGAATTCACCACCTCTTAG Template: pDL189 HindIII/EcoRI cloned into pDL290 HindIII/EcoRI cut again HindIII/XbaI cloned into pCDNA3.1Hygro-3HA HindIII/XbaI	HindIII/ XbaI
AUP1-ΔCUE-UbK48R-D58A-HA	pDL311	pCDNA3.1Hygro-3HA ampicillin	Ub-D58A-for CTGTCTGCCTACAACATC Ub-D58A-rev GATGTTGTAGGCAGACAG Ub-noStop-XbaI-rev and AUP1_HindIII_for Template: pDL263	HindIII/ XbaI
AUP1-ΔCUE-UbI44A-K48R-HA	pDL314	pCDNA3.1Hygro-3HA ampicillin	Ub-I44AonK48R-for CAGAGGTTGGCCTTTGCTGG Ub-I44AonK48R-rev CCAGCAAAGGCCAACCTCTG Ub-noStop-XbaI-rev and AUP1_HindIII_for Template: pDL263	HindIII/ XbaI
AUP1-10KR-HA	pDL366	pCDNA3.1Hygro-3HA ampicillin	See methods: 3.2.9. DNA amplification and modification by PCR	HindIII/ XbaI
AUP1-10KR-UbK48R-HA	pDL368	pCDNA3.1Hygro-3HA ampicillin	AUP1_HindIII_for and AUP1_nostop_EcorI_rev Template: pDL366 HindIII/EcoRI cloned into pDL189 HindIII/EcoRI cut again HindIII/XbaI cloned into pCDNA3.1Hygro-3HA HindIII/XbaI	HindIII/ XbaI

Constructs with alias pCT, pWZ and pAS were kindly supplied by Christoph Thiele, Wolfgang Zachariae and Ana Stevanovic, respectively.

Constructs pCT7, pCT15 and pDL211 were described before (Spandl *et al.*, 2011).

Constructs pCT116 and pCT117 were described (Spandl Johanna, PhD thesis 2009).

pCDNA3.1Hygro-3HA was used as control when indicated.

3.1.3. Antibodies

Table 2: List of primary antibodies

Name/antigen	Species	Dilution	Producer
AUP1/ancient ubiquitous protein 1	Rabbit	1:5000 (WB) 1:400 (IF)	Christoph Thiele, cited in (Spandl *et al.*, 2011)
HA-F7/Influenza hemagglutinin	Mouse	1:1000 (WB) 1:100 (IF)	Santa cruz, 7329
HA-Y11/Influenza hemagglutinin	Rabbit	1:1000 (WB)	Santa cruz, 805
Myc-9E10/C-myc	Mouse	1:1000 (WB)	Santa cruz, 40
Myc-A-14/C-myc	Rabbit	1:1000 (WB)	Santa cruz, 789
ACSL3/acyl-CoA synthetase long-chain family member3	Rabbit	1:2500 (WB)	Christoph Thiele, cited in (Spandl *et al.*, 2011)
NSDHL/NAD(P)H steroid dehydrogenase-like protein	Rabbit	1:2500 (WB)	Christoph Thiele, cited in (Moessinger *et al.*, 2011)
GAPDH (1D4)/glyceraldehyde-3-phosphate dehydrogenase	Mouse	1:1000 (WB)	NovusBiologicals, NB300-221

3.2. Methods

3.2.1. Cell culture

COS-7 and A431 cell line were from ATCC (Virginia, US) with ID numbers CRL-1651 and CRL-1555, respectively. COS-7 and A431 cells were grown in a humidified incubator with 5% CO_2 at 37°C and cultured in DMEM (Gibco, 41965) supplemented with 10% FCS (Gibco, 10437). Cells were split regularly at around 80% confluency with trypsin/EDTA (Gibco, 25300).

3.2.2. Thawing of cells

Vials containing frozen cells were thawed in a water bath set at 37°C. Then, cells were transferred into a new culture flask and DMEM + 10% FCS was added. Cells were kept in the humidified incubator and split at 80% confluency.

3.2.3. DNA transfection

For transfection in 24-well and 6-well plates, cells were grown until reaching a confluency of around 70%, washed with PBS and 200 µl Opti-MEM (Gibco, 11058) were added to each well of a 24-well or 1ml to each well of a 6-well plate and kept in the humidified incubator.

3-4 µl Lipofectamine 2000 (Life Technologies, 11668-019) was mixed with 100 µl Opti-MEM, and 1 µg of plasmid DNA was mixed with another 100 µl of Opti-MEM. Both mixes were incubated for 5min at room temperature and then both mixes were combined and incubated for another 20min. From this transfection mix, 40 µl per well of a 24-well or 200 µl per well of a 6-well plate were added drop wise to the prepared cells. 4h post transfection medium was replaced by 1 ml per well of a 24-well or 3 ml per well of a 6-well plate of fresh DMEM + 10% FCS supplemented with 50 µM oleate. For analysis of cellular proteins or analysis by microscopy cells were harvested or fixed approximately 24h post transfection.

3.2.4. siRNA transfection

Approximately 5000 A431 cells were plated per well of a 24-well plate in 1 ml DMEM + 10% FCS the day before transfection. The next day cells were washed with PBS and 200 µl Opti-MEM were added to each well of the 24-well plate. 0.8 µl Lipofectamine 2000 was mixed with 50 µl Opti-MEM, and 2 µl siRNA (20 µM) (see 3.1.3.) was mixed with another 50 µl Opti-MEM. Both mixes were incubated for 5min at room temperature and then both mixes were combined and incubated for another 20min. The entire transfection mix was added drop wise to the prepared cells. Cells were incubated over night and then the transfection medium was replaced by 1ml per well of a 24-well plate of fresh DMEM + 10% FCS supplemented with 50 µM oleate. For analysis of cellular proteins or analysis by microscopy cells were harvested or fixed approximately 72h post transfection.

3.2.5. Cultivation of *E. coli*

E. coli were grown in LB-Medium or on LB-Agar plates (LB-Medium: 5 g/L yeast extract, 10 g/L NaCl, 10 g/L tryptone, containing ampicillin (amp) (100 µg/ml) or kanamycin (kan) (50 µg/ml) at 37°C shaking at 180rpm or without shaking for cells grown on LB-Agar plates.

3.2.6. Preparation of chemical competent *E. coli*

E. coli DH5-alpha cells were grown in 250 ml SOB Medium (SOB Medium: 0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) at 19°C to OD 0.5. Then cells were put on ice for 10min. Bacteria were pelleted by centrifugation (4000g/10min/4°C) and resuspended in 16 ml Transformation Buffer (Transformation Buffer: 10 mM PIPES, 250 mM KCl, 15 mM $CaCl_2$, 55 mM $MnCl_2$, adjusted to pH 6.7 with KOH (before the addition of $MnCl_2$)). Cells were again pelleted and resuspended in 20 ml Transformation Buffer supplemented with 1.4 ml DMSO. 100 µl aliquots were shock frozen in liquid nitrogen and stored at -80°C.

3.2.7. Transformation of *E. coli*

Chemical competent bacteria were thawed on ice and mixed with 50 ng of plasmid DNA or the entire DNA from a ligation mix. Bacteria were kept on ice for 5min followed by a heat shock at 42°C for 60sec. Afterwards, bacteria were plated directly onto LB plates, supplemented with amp or 1 ml LB-Medium was added and cells kept at 37°C for 1h, pelleted, resuspended and then plated onto LB plates supplemented with kan.

3.2.8. DNA preparation from *E. coli*

DNA was prepared according to manufacturers (Macherey-Nagel) protocols as Mini or Midi preparations. DNA Concentration was determined using the NanoDrop photospectrometer. Midi and Maxi preparations were diluted to final concentration of 1 µg/µl with dH_2O. DNA sequencing was performed by GATC-Biotech GmbH.

3.2.9. DNA amplification and modification by PCR

DNA was amplified from plasmids with pre-designed primers (see table 1) using standard lab PCR protocols. SYBR green I (Invitrogen, S7567) was added to PCR amplified DNA to a final dilution of 1:15000 and loading dye according to manufactures (New England BioLabs) protocol. PCR products were separated on 1% agarose TAE (TAE (50x): 100 ml 0.5 M Na_2EDTA pH 8.0, 242 g Tris base, 57.1 ml glacial acetic acid, H_2O up to 1000 ml) gels. PCR products were extracted from gels according to manufactures (Qiagen) instructions and digested with appropriate restriction enzymes (see table 1) according to manufactures (New England BioLabs) protocols. Digested PCR products were purified over and extracted from agarose TAE gels. Digested PCR products were ligated into appropriate DNA plasmids (pre-digested with appropriate restriction enzymes) (see table 1) using standard lab protocols (T4-DNA ligase and ligation buffer were obtained from MPI-Dresden in-door facility).

For the introduction of point-mutations by PCR, the following approach was chosen. Forward and reverse Primers containing the appropriate mismatches and spanning the identical region where point-mutations should be introduced were designed. Two independent PCR reactions with either forward or reverse primer were performed with appropriate complementary forward and reverse primers (see table 1). Both PCR products were purified over and extracted from agarose TAE gels and combined for an additional PCR reaction. PCR reaction was set up using standard lab PCR protocols with the following modifications.

PCR was run for 5 cycles and then the same complementary forward and reverse primers as used before in the two independent PCR reactions were added and PCR ran for another 30 cycles. Melting temperature was set for 25sec and annealing temperature was set for 25sec. Finally, PCR products were purified and extracted from agarose TAE gels, digested, purified and extracted again from agarose TAE gels and ligated into appropriate DNA plasmid (protocol adapted from Ho et al., 1989 (Ho *et al.*, 1989)).

For sequential DNA digestion, DNA was first digested to completion with the first restriction enzyme having only one recognition site. Digestion products were split into three tubes. Second restriction enzyme, having two recognition sites, was added to each tube in decreasing concentrations and digestion was carried out for 1min (for

HighFidelity restriction enzymes). DNA was immediately loaded on 2% agarose TAE gels and appropriate band cut from gel and purified.

Cloning of AUP1-10KR (AUP1-K143R-K250R-K264R-K269R-K305R-K322R-K347R-K359R-K377R-K390R-HA): The above-described protocols were used to introduce additional lysine to arginine mutations into pDL91 (see table 1), mutating one lysine residue to arginine for every complete cloning round (Primers used as in table 1). The generated construct was then used for the next cloning round. The two most C-terminally located lysine residues were mutated to arginine by two sequential cloning rounds using Primers: AUP1-HindIII-for and AUP1-rev (see table 1) together with the following primers:

AUP1-K377-for: CATTTGCCAGCTCTTCCTGG together with

AUP1-K377-for: CCAGGAAGAGCTGGCAAATG

and

AUP1-K390-for: CAGGAGCGCAGGCAAGCAC together with

AUP1-K390-rev: GTGCTTGCCTGCGCTCCTG

3.2.10. SDS poly-acrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared in SDS Sample Buffer (Laemmli Buffer (5x): 125 mM Tris-HCl pH 6.8, 4% SDS, 0.004% Bromophenol blue, 20% (w/v) glycerol, 2% betamercaptoethanol).

Mini-protean system from Bio-Rad was used to cast and subsequently run SDS-PAGE gels. Standard lab protocols were used and the following stock solutions: 1x Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS), 4x Separating Gel Buffer (1.5 M Tris-HCl pH 8.8, 0.4% (w/v) SDS), 4x Stacking Gel Buffer (0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS), 30% acrylamide stock solution (30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide), TEMED and 10% (w/v) ammonium persulphate. Gels were blotted or stained in Coomassie blue solution (0.25% (w/v) Coomassie Brilliant Blue R250, 40% methanol, 10% glacial acetic acid, 50% dH_2O) shaking at RT for 1-4h and destained using destaining solution (50% methanol, 10% glacial acetic acid, 40% dH_2O). Gel was scanned after complete destaining procedure.

3.2.11. Western blot (WB)

Proteins were blotted onto Nitrocellulose membranes "Protran" (Whatman, Dassel, Germany) using Mini-protean system from Bio-Rad and Blotting Buffer (400 ml 1x Running Buffer, 200 ml methanol, 400 ml dH_2O) at 350mA for 1h. Membranes were blocked (Blocking Buffer: PBS containing 0.05% Tween-20 and 5% milk powder) for 0.5-1h at RT or over night at 4°C. After blocking, appropriate primary antibodies were diluted (1:1000 to 1:5000) in Blocking Buffer and added onto the membrane and incubated 1h at RT or over night at 4°C shaking. Membranes were washed 2-3 times with Blocking Buffer and incubated with HRP (Horseradish peroxidase)-conjugated secondary antibody (Jacksons Immunological, West Grove, PA) (1:5000 in Blocking Buffer) for 1h. Finally membranes were washed 3x with Blocking Buffer, 2x with PBS containing 0.05% Tween-20 and 2x with PBS, each 10min. Detection was done using QImaging Fast 1394 Rolera-Mgi Plus camera controlled by Gel-Pro Analyzer Software (Media Cybernetics, Inc, Bathesda, MD, USA).

In order to strip the antibodies from a WB membrane to reprobe with a different antibody; the membrane was incubated with a 1x PBS solution containing 1% SDS and 3 mg of TCEP. The membrane was then incubated for 20min at 60°C in a water bath. Afterwards the membrane was washed two times for 5min with 1x PBS with 0.1% TWEEN and reprobed with new antibodies.

3.2.12. Crude cell lysates

After washing cells with PBS, cells were either lysed directly in 2x Sample Buffer or lysed with Mild Lysis Buffer (Mild Lysis Buffer: 25 mM HEPES pH7.5, 150 mM NaCl, 1% Triton-X100, 0.5% Na-deoxycholate) and 5x SDS Sample Buffer was added to 2x final concentration. After boiling at 95°C for 5-10min samples were resolved by SDS-PAGE.

3.2.13. Chloroform-methanol precipitation

Protein precipitation followed the method described by Wessel and Fluegge, 1984 (Wessel and Flügge, 1984). For sample volumes of 100 µl, 400 µl of 2:1 methanol:chloroform was added, then 100 µl dH_2O and 100 µl chloroform. Sample

was mixed vigorously and centrifuged (10000g/10min/RT). Upper phase was removed and 500 µl methanol was added and sample was centrifuged (10000g/10min/RT). Supernatant was removed, the pellet air dried and dissolved in 2x Sample Buffer.

3.2.14. Analysis of AUP1 expression levels

For analysis of the expression levels of AUP1, COS-7 and A431 cells were plated in 6-well plates. An equal number of cells were lysed from one well containing COS-7 or A431 cells in Mild Lysis Buffer (see 3.2.12.) and proteins were Chloroform-methanol precipitated (see 3.2.13.). Proteins were re-dissolved in 2% SDS solution. For protein determination 50µL of fluorescamine solution (25 mg fluorescamine in 100 ml acetone), 50 µL of protein solution and 150 µL of 0.1 M sodium borate buffer with pH 9 was mixed and added to a 96 well plate. Then fluorescence was determined using the Tecan i control infinite pro200 with 360 nm, 35 nm bandwidth excitation filter and a 465 nm, 35 nm bandwidth emission filter.

For calibration a BSA dilution series was used. Protein concentration was equalized across all samples with 2% SDS solution and equal amounts from all three samples were mixed with equal volumes of 5x Sample Buffer (to final concentration of 2x Sample Buffer). Proteins from equal volumes from both samples were separated on SDS-PAGE and AUP1 detected with the AUP1 specific antibody (see table 2).

3.2.15. Immunoprecipitation of myc-tagged AUP1

Cells were plated in 6-well plates and seeded at a density of approximately 60%, transfected the next day with myc- and HA-tagged AUP1 plasmids (see table 1) using Lipofectamine 2000 and grown for 24h in DMEM + 10% FCS supplemented with 50 µM oleate followed by immunoprecipitation as detailed below.

Cells were washed with ice cold PBS and lysed in 1 ml ice cold Lysis Buffer (Lysis Buffer: 25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40 and cOmplete Protease inhibitor cocktail without EDTA (Roche, 12936900). Lysis was performed at 4°C for 5-10min shaking. Lysate was centrifuged (15000g/5min/4°C) and 100 µl saved as 10% input and chloroform:methanol precipitated. To the remaining 900 µl, 10 µl anti-

myc specific antibody (Myc-9E10, mouse) and 20 µl Protein-A sepharose (CL4B, GE Healthcare) was added. Samples were incubated at 4°C over night on rotary wheel, centrifuged (1000g/1min/4°C) and Protein-A sepharose washed three times with Lysis Buffer followed by elution with 80 µl glycine pH 2.2. Then, 20 µl of 2M Tris-HCl pH 8.8 was added immediately to the eluate followed by 25 µl 5x Sample Buffer. 35 µl from 10% input and from eluate were loaded on 10% SDS-PAGE. Immunostaining was done against HA-tag (HA-Y11, rabbit), myc-tag (Myc-A-14, rabbit), each on separate WB membranes. For Immunostaining against ACSL3 and NSDHL membrane was cut at around 60kDa and upper part immunostained with anti-ACSL3 and lower part with anti-NSDHL antibody. Antibodies were stripped from membrane were indicated, membrane was then cut at around 60kDa and upper part immunostained with anti-ACSL3 and lower part with anti-NSDHL antibody.

3.2.16. Ubiquitination-assay

Cells were plated in 6-well plates and seeded at a density of approximately 60% and transfected the next day with His-tagged ubiquitin (0.5 µg/100 µl Opti-MEM was used) and HA-tagged AUP1 plasmids (see table 1) using Lipofectamine 2000 (see 3.2.3.) Cells were grown for 24h in DMEM + 10% FCS supplemented with 50 µM oleate followed by His-tag purification as detailed below.

Cells were washed with PBS and lysed in 1 ml Lysis Buffer (Lysis Buffer: 6 M Guanidine hydrochloride, 100 mM disodium hydrogen phosphate pH 7.4, 0.5% Triton-X100, 20 mM imidazole, 10 mM Tris-HCl pH 7.4). 100 µl of cell lysate was kept for 10% input and chloroform-methanol precipitated. 35 µl of 50% slurry of Ni-Agarose (Ni-NTA Superflow, Qiagen) was added to remaining cell lysate and incubated at rotary wheel at RT for 2-3h, centrifuged (1500g/1min/RT) and Ni-Agarose was washed three times with Washing Buffer (Washing Buffer: 8 M urea, 100 mM disodium hydrogen phosphate pH 7.4, 0.5% Triton-X100, 20 mM imidazole, 10 mM Tris-HCl pH 7.4) followed by elution with 50 µl Elution Buffer (Elution Buffer: 250 mM imidazole, 150 mM NaCl, 25 mM Tris-HCl pH 6.0). Finally, 5x SDS Sample Buffer was added to eluate to a 2x final concentration. Proteins were separated on 10% SDS-PAGE and immunoblotted with anti-HA antibody (HA-F7).

3.2.17. Paraformaldehyde fixation of cells for microscopy

Cells were washed with PBS and fixed in PBS with 3.7% (v/v) paraformaldehyde (PFA). After 30min the PFA-mix was removed and cells were washed with PBS. Fixed cells were stored in PBS at 4°C and stained the next day.

3.2.18. Immunofluorescence microscopy

For immunofluorescence microscopy, fixed cells were blocked in Blocking Buffer (Blocking Buffer (BB): PBS with 1% BSA and 0.2% saponin) for 30min. Incubation with 1st antibody was performed on parafilm onto 30 µl of diluted primary antibody (HA-F7 antibody diluted 1:100 and AUP1 antibody diluted 1:500) in BB for 1h. Cells were washed 3x 10min with BB in 24-well plate and incubated with 30 µl secondary antibody (conjugated to Alexa 488 or 647 (Life Technologies)) diluted 1:500, again on parafilm. Cells were washed 3x 10min with BB in 24-well plate and DAPI and LD540 stained as detailed below.

100 µl of PBS containing DAPI (1µg/ml in PBS) and LD540 (Spandl *et al.*, 2009) were added to each well of the 24-well plate and incubated for 15min, shaking and protected from light. Cells were washed 3x 10min with PBS and 1x with dH_2O and mounted with 5 µl mowiol/DABCO (6 g glycerol, 2.4 g mowiol, 6 ml dH_2O, 12 ml 0.2 M Tris-HCl pH 8.5, 0.1% DABCO) on microscope slide. Images of fixed samples were acquired using Zeiss Axio Observer.Z1 equipped using 63x/NA 1.4 Oil Plan-Apochromat or 63x/NA1.4 objectives.

3.2.19. ZFNs working protocol: mRNA preparation

In principal, ZFNs delivery into cells and analysis of putative knock-out cells were done according to manufactures (Sigma-aldrich) protocols, using AUP1 specific ZFNs generated by Sigma-aldrich (CompoZR Knockout Zinc Finger Nucleases, CKOZFND3771-1KT). The ZFN binding and cutting site is as follows with putative cutting site in bold and underlined:

GACCATGTGTGCGGT**GCTAG**GGCTCGTGGCCCGGCAGGA

The following kits were used:

To efficiently deliver ZFNs coding sequence into cells they were first in vitro transcribed into mRNA. mRNA was generated independently for both AUP1 specific ZFNs. The linearization of the plasmid DNA was accomplished via standard restriction digest using XbaI restriction enzyme to generate 5´-protruding ends. Generation of mRNA was done using the MessageMAX™ T7 ARCA-Capped Message Transcription Kit (150482, Biozym, Hessich Oldendorf). poly(A) tailing was done using A-Plus Poly(A) Polymerase Tailing Kit (15491, Biozym, Hessich Oldendorf). The mRNA was purified using the MEGAClear Kit (AM1908, LifeTechnologies). The mRNA content was measured using NanoDrop photospectrometer. Both mRNAs resulting from each ZFN were combined in an equal molar ratio.

3.2.20. ZFNs working protocol: Transfection of cells

A431 cells were seeded in 6-well plates. Transfection was done using Trans-IT mRNA Transfection Kit (MIR2225, MoBiTec Distributor of Mirus in germany, Göttingen) according to manufactures instructions.

3.2.21. ZFNs working protocol: Single cell sorting

Transfected cells were trypsinated 72h post-transfection and centrifuged. Cells were single sorted in 96-well plates by Marcus Beier (using FACS sorter). Single clones were propagated and genomic DNA was isolated using GenElute Mammalian Genomic DNA Miniprep Kit (G1N70, Sigma-Aldrich). Genomic DNA was analyzed for successful genomic alteration using the CelI assay.

3.2.22. ZFNs working protocol: CelI assay

The region were ZFNs are thought to introduce alterations into the genomic DNA of AUP1 were amplified using forward and reverse primers supplied by Sigma-Aldrich (ZFN Primer F: GGATCCACGTCTTCCTGGT and ZFN Primer R: GGGTTAGGGGTAAGGCTCAG) and standard lab PCR protocols. PCR products were purified and extracted from agarose TAE gels. Elution was done with Elution Buffer (Elution Buffer: 12,5 µl 2M KCl, 2,5 µl 2 M Tris-HCl pH 7,5, 50 µl 0,15 M

$MgCl_2$, according to CelI manufactures protocols). 400 ng of the purified PCR product were taken directly for the annealing assay. Assay was done in the Thermocycler using PCR tubes with the following Program:

95ºC 10 min; 95ºC to 85ºC (-2.0ºC/s); 85ºC 1 min; 85ºC to 75ºC (-0.3ºC/s); 75ºC 1 min; 75ºC to 65ºC (-0.3ºC/s); 65ºC 1 min; 65ºC to 55ºC (-0.3ºC/s); 55ºC 1 min; 55ºC to 45ºC (-0.3ºC/s); 45ºC 1 min; 45ºC to 35ºC (-0.3ºC/s); 35 ºC 1 min; 35ºC to 25ºC (-0.3ºC/s); 25ºC 1 min; 4ºC Hold.

The PCR product was then supplemented with 1µl of SURVEYOR-Nuclease and 1µl of the Enhancer-Solution, both from the Transgenomic SURVEYOR Mutation Detection Kit (706020, Transgenomic Inc.). The digestion was done at 42 ºC for one hour using the Thermocycler.

4. Results

4.1. AUP1 overexpression causes clustering of LDs

To investigate the role of AUP1 in the intracellular distribution of LDs, AUP1 was overexpressed in one of our standard cell lines, the monkey kidney fibroblast COS-7 cell line. COS-7 cells readily induced the formation of LDs, which were usually dispersed throughout the cells (see Introduction Fig. 3, A), when supplemented with oleate.

Overexpression of HA-tagged AUP1 (AUP1-HA) in COS-7 cells caused a striking change in the intracellular distribution of LDs. In COS-7 cells overexpressing AUP1-HA, LDs accumulated in one or few LD clusters whereas the cells were almost devoid of any solitary LD (Fig. 9, A). In contrast, COS-7 cells transfected with an empty control vector showed no LD clustering whereas numerous LDs were dispersed throughout the cells (Fig. 9, B). Next, it was analyzed whether the observed clustering of LDs in AUP1 overexpressing cells is quantifiable. As LD clustering was readily detectable upon overexpression of AUP1, a cell was simply defined to show LD clustering when the majority of LDs were packed in LD clusters and only few dispersed LDs could be observed. About 80% of cells overexpressing AUP1-HA showed LD clustering whereas only 5% of cells transfected with empty control vector exhibited LD clustering (Fig. 13, A). These results suggest that AUP1 actively promotes the aggregation of LDs to form one or few LD clusters.

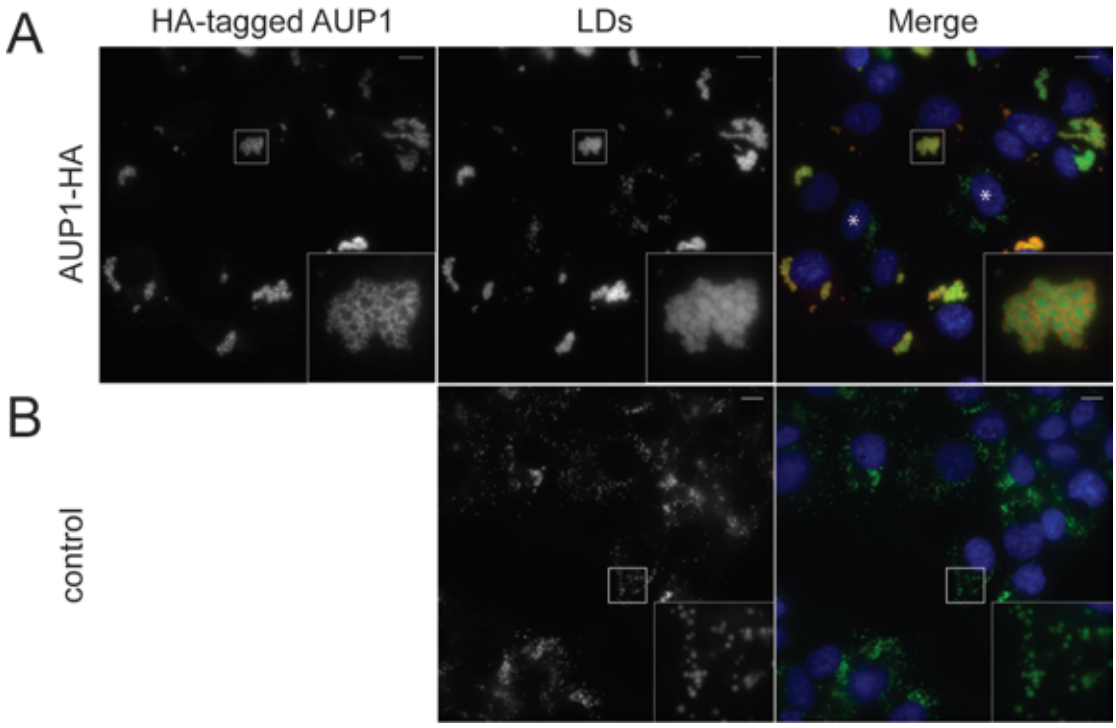

Figure 9.

AUP1 overexpression causes LD clustering

(A) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1 and supplemented with 50 µM oleate are shown. Cells were immunostained with anti-HA antibody (left panel), LDs were stained with LD540 (middle panel). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panel). Bars, 10µm. Note: Cells not expressing AUP1 do not show LD clustering (marked with asterisk (*)). (B) COS-7 cells transfected with an empty control vector and supplemented with 50 µM oleate. LDs were stained with LD540 (middle panel). Merged image with nuclei stained by DAPI in blue and LDs in green are shown (right panel). Bars, 10µm.

4.2. Expression levels of AUP1

AUP1 was found to be highly abundant in a purified LD fraction from the human epithelial A431 cell line (Moessinger *et al.*, 2011). Furthermore, it was observed that A431 cells form LD clusters when supplemented with oleate (see Introduction Fig. 3, B). Hence, different expression levels of AUP1 in A431 cells compared to COS-7 cells might explain the observed difference in the intracellular distribution of LDs.

Equal amounts of protein from COS-7 and A431 cells were separated on SDS-PAGE and immunoblotted with anti-AUP1 antibody. The A431 cells contained relatively

high levels of AUP1 compared to COS-7 cells (Fig. 10). These results indicate that the difference in endogenous AUP1 expression between these two cell lines could be responsible for the absence of LD clusters in COS-7 cells and the formation of LD clusters in A431 cells.

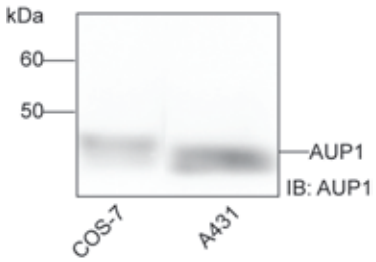

Figure 10.

Relative expression levels of AUP1

Relative expression levels of endogenous AUP1 in COS-7 and A431 cells. Equal amounts of protein from COS-7 and A431 cells were separated on SDS-PAGE and immunoblotted with anti-AUP1 antibody.

4.3. Knock-down of AUP1 in A431 cells causes declustering of LDs

Results so far indicate that AUP1 is important for the clustering of LDs in cells and that the relatively high expression levels of AUP1 observed in the A431 cell line might be responsible for the strong tendency of A431 cells to form LD clusters.

Hence, a knock-down approach was used to investigate whether the clustering of LDs in A431 cells depends on endogenous expression levels of AUP1. Three different stealth siRNAs against AUP1 were analyzed with respect to their knock-down efficiency in A431 cells. Two of three siRNAs showed a strong reduction of endogenous AUP1 levels (Fig. 11, A). These two siRNAs were used to knock-down AUP1 in A431 cells and LD clustering was analyzed and compared to mock transfected A431 cells. Knock-down of AUP1 with either siRNA strongly decreased LD clustering compared to the control cells (Figure 11, B and C), suggesting that AUP1 is involved in the clustering of LDs in the A431 cell line. These results further

indicate that endogenous expression levels of AUP1 can be sufficient to induce LD clustering.

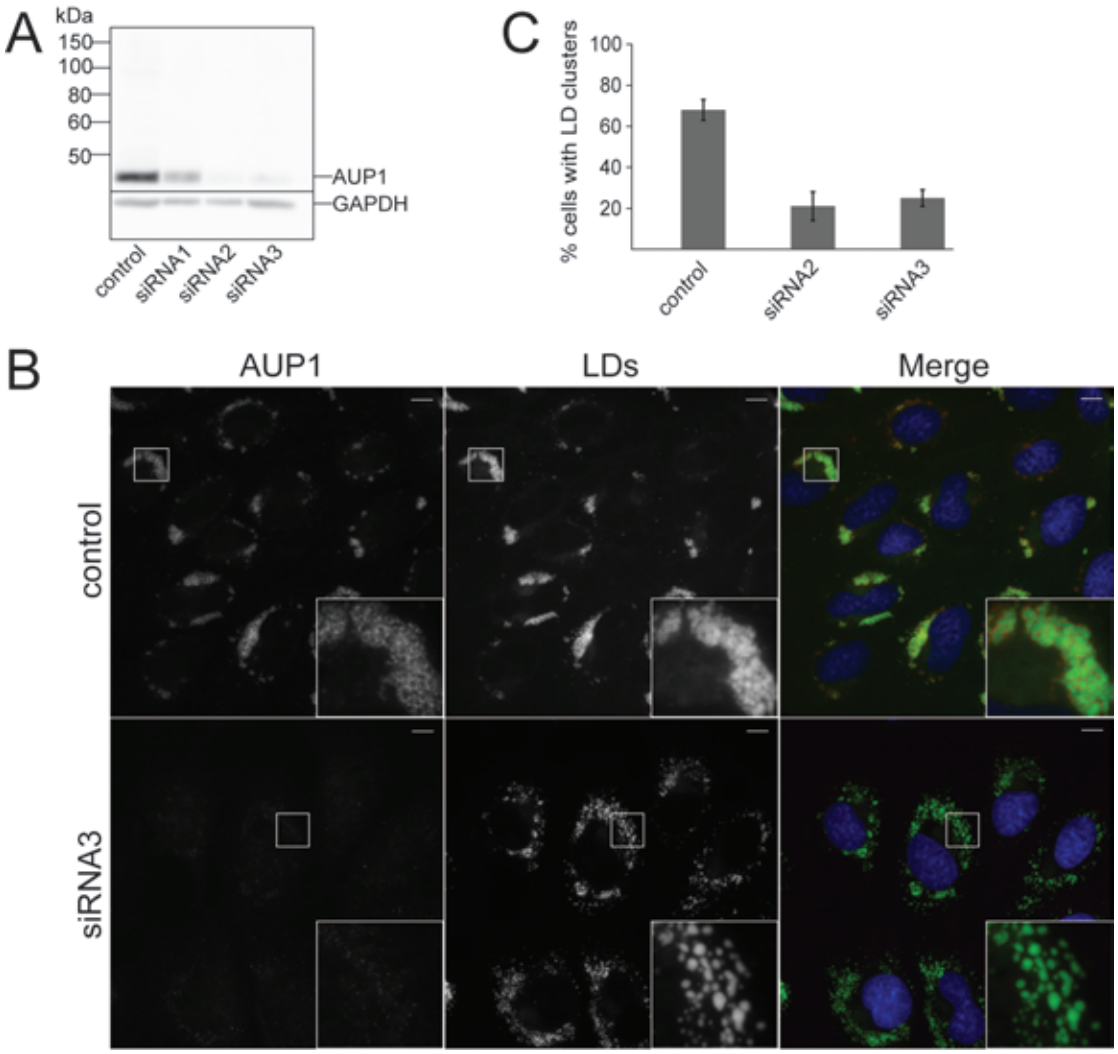

Figure 11.

Knock-down of AUP1 causes declustering of LDs

(A) A431 cells were either mock transfected or transfected with one of three different siRNAs against AUP1. Cells were lysed and proteins separated on SDS-PAGE and immunoblotted with anti-AUP1 antibody. GAPDH served as loading control. (B) Fluorescence micrographs of A431 cells either mock transfected (control) or transfected with siRNA3 and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-AUP1 antibody (left panels), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm. (C) Quantification of LD clustering in A431 cells mock (control) or siRNA (as indicated) transfected. Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed.

4.4. The AUP1 CUE domain is important for LD clustering

To identify functional domains of AUP1 important for LD clustering we generated truncation and deletion mutants of AUP1 fused to an HA-tag and studied their effect on LD clustering. We focused on the two functional domains located at the C-terminus of AUP1, the G2BR and CUE domain. The G2BR domain is essential for binding Ube2g2 (Spandl *et al.*, 2011; Klemm *et al.*, 2011). The CUE domain belongs to a group of UBDs, which have been shown to interact with ubiquitin (Shih *et al.*, 2003).

Overexpression of AUP1 (1-362) lacking the G2BR domain (AUP1-ΔG2BR-HA) still caused clustering of LDs in COS-7 cells (Fig. 12, A), with around 85% of cells showing LD clustering (Fig. 13, A). In contrast, overexpression of an internal deletion mutant of AUP1 (Δ295-339) lacking the CUE domain (AUP1-ΔCUE-HA) in COS-7 cells did not induce LD clustering as strongly as was observed for the overexpression of AUP1-HA (for the sake of simplicity: similar observations in COS-7 cells are from now on referred to as "attenuated LD clustering"), (Fig. 12, B). Only around 30% of cells analyzed showed LD clustering (Fig. 13, A), suggesting an important role for the CUE domain in LD clustering. The CUE domain consists of three alpha-helices with conserved residues for binding ubiquitin on helices one and three and key residues for the helix one and two packing interface on helix two (Fig., 13, C) (Kang *et al.*, 2003; Prag *et al.*, 2003). We mutated amino acid residues within these conserved regions to hinder interaction with ubiquitin. Figure 13, C shows the AUP1 CUE mutants generated. Comparable to the AUP1-ΔCUE-HA construct, overexpression of these AUP1 CUE mutants in COS-7 cells strongly attenuated LD clustering (Fig. 12, C and D; Fig. 13, A), confirming the importance for the CUE domain in LD clustering. Importantly, truncation of the G2BR domain and deletion or mutation of the CUE domain did not change the localization of AUP1 to LDs (Fig. 12, A- D, inset).

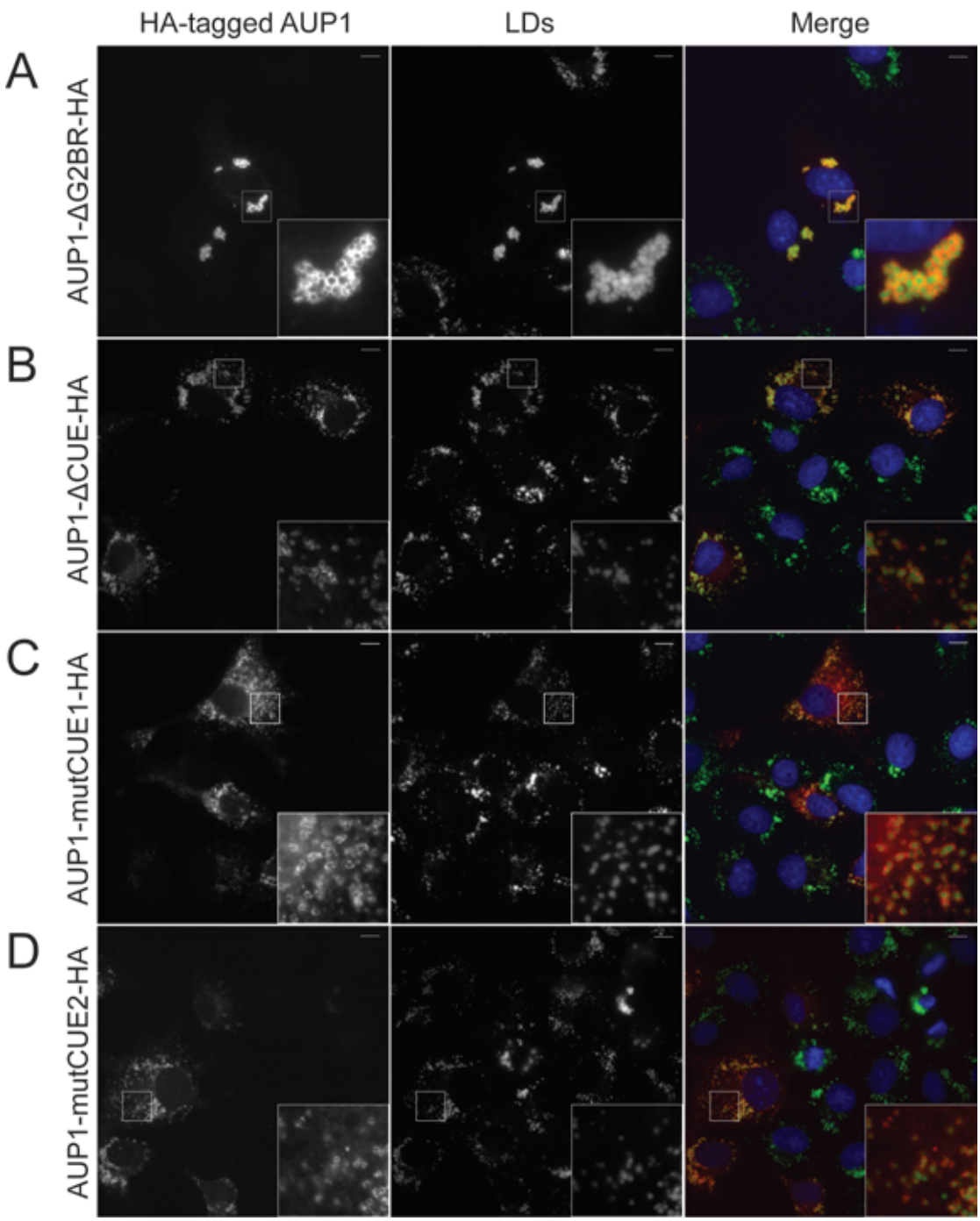

Figure 12.

The AUP1 CUE domain is important for LD clustering

(A-D) Fluorescence micrographs of COS-7 cells overexpressing different HA-tagged AUP1 constructs (as indicated) and supplemented with 50 μ M oleate are shown. Cells were immunostained with anti-HA antibody (left panels), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm.

To test whether different expression levels of the above mentioned constructs might be responsible for the observed difference in LD clustering, these AUP1 constructs were overexpressed in COS-7 cells and expression levels analyzed by immunoblotting against the HA-tag, using GAPDH as loading control (Fig. 13, B). AUP1-ΔCUE-HA and AUP1-mutCUE3-HA had higher expression levels than AUP1-HA, but strongly attenuated LD clustering. On the other hand, AUP1-ΔG2BR-HA as well had higher expression levels than AUP1-HA, but promoted LD clustering to comparable extends. These results suggest that attenuated LD clustering in COS-7 cells overexpressing AUP1 with a deleted or mutated CUE domain does not result from a difference in expression levels compared to AUP-HA.

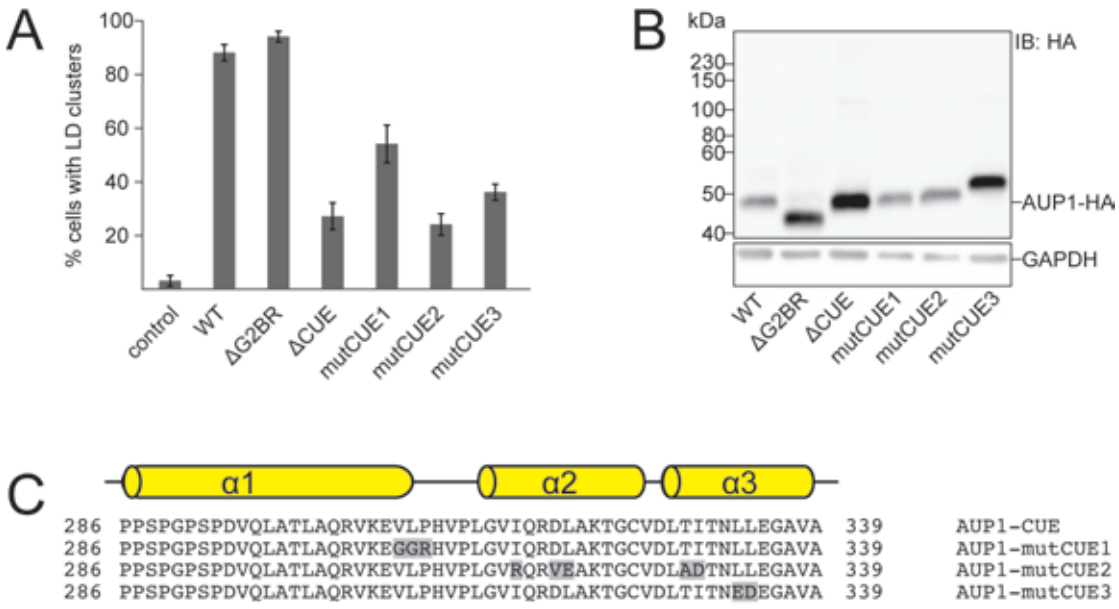

Figure 13.

The AUP1 CUE domain is important for LD clustering II

(A) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Empty vector was used as control. Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed. (B) Expression levels of HA-tagged AUP1 constructs. Proteins from COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated) were separated on SDS-PAGE and immunoblotted with anti-HA antibody. GAPDH served as loading control. Note: AUP1-ΔCUE and AUP1-mutCUE3 migrated at an apparent molecular weight around five kDa higher than expected. (C) Amino acid sequence of the AUP1 CUE domain and predicted relative position of the three α-helices after Prag et al. (Prag *et al.*, 2003). Mutated amino acid residues of the three AUP1-mutCUE constructs used in this work are highlighted in grey.

4.5. AUP1 is ubiquitinated in COS-7 cells

It has been observed that AUP1 is ubiquitinated in HeLa cells (Klemm *et al.*, 2011). It was therefore analyzed whether AUP1 is also ubiquitinated in COS-7 cells, employing the His-tag affinity purification.

COS-7 cells were transfected with His-ubiquitin and AUP1-HA followed by solubilization with 6 M guanidine hydrochloride. His-ubiquitin and any His-ubiquitin modified proteins were recovered from cell lysate over Ni-NTA agarose and immunoblotted against the HA-tag of AUP1. Two major bands for AUP1-HA were detected, migrating at an apparent molecular weight of 8 and 16 kDa above the expected molecular weight for AUP1-HA (Fig. 14). This molecular weight shift suggests a modification of AUP1 by one and two ubiquitin moieties, respectively.

Next, the same His-tag affinity purification was used to analyze whether any of the before described AUP1 mutations obviate the ubiquitination of AUP1.

For AUP1-ΔG2BR-HA, the same molecular weight shift was observed as for AUP1-HA (Fig. 14). In contrast, this molecular weight shift was almost undetectable for AUP1-ΔCUE-HA or for the HA-tagged AUP1 CUE mutants (Fig. 14), demonstrating that the CUE domain of AUP1 is essential for AUP1 ubiquitination in COS-7 cells, whereas the G2BR domain is dispensable for this process. In addition, a high molecular weight smear was detectable for both, AUP1-HA and AUP1-ΔG2BR-HA (Fig. 14), suggesting that AUP1 protein levels are also regulated posttranslational through polyubiquitination.

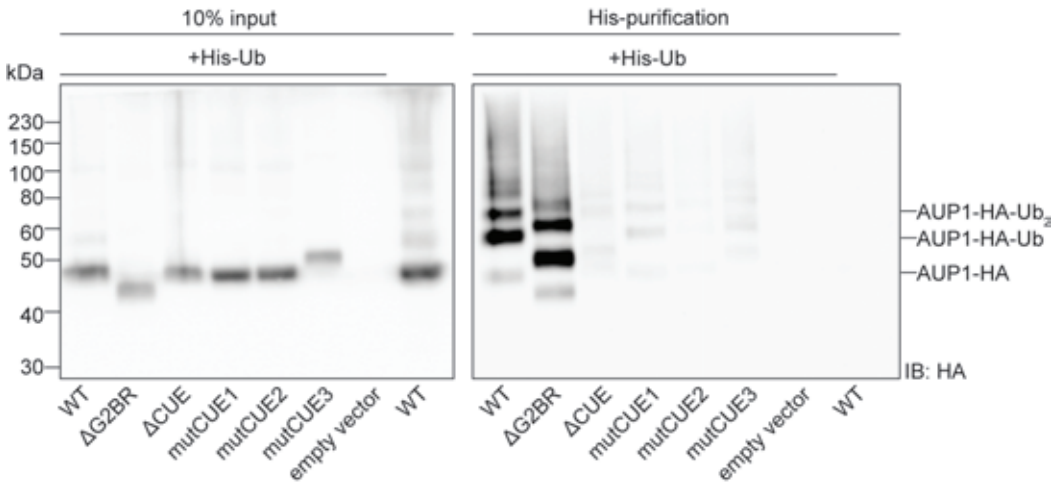

Figure 14.

AUP1 is ubiquitinated in a CUE dependent manner

COS-7 cells were transfected with His-ubiquitin and HA-tagged AUP1 constructs and controls (as indicated). His-ubiquitin and His-ubiquitin modified proteins were purified from cell lysates over Ni-NTA agarose. Proteins from lysates (10% input) and His-purifications were separated on SDS-PAGE and immunoblotted with anti-HA antibody.

4.6. AUP1 is ubiquitinated on multiple lysine residues

Ubiquitination usually leads to the formation of a covalent bond between ubiquitin and a lysine residue of the target protein. AUP1 contains ten lysine residues representing potential targets for ubiquitination. To investigate which lysine residues are important for ubiquitination, single lysine to arginine (K-to-R) mutations were introduced into HA-tagged AUP1. The two most C-terminally located lysine residues, Lys377 and Lys390 were not analyzed this way. Both, Lys377 and Lys390 are deleted in the AUP1-ΔG2BR-HA construct, which showed the same ubiquitination pattern as AUP1-HA (Fig. 14). These lysine residues were therefore not considered to be potential targets for ubiquitination.

As shown in Figure 15, A each AUP1 construct containing single K-to-R mutations exhibited the same ubiquitination pattern as AUP1-HA, suggesting that AUP1 can be ubiquitinated on more than one lysine residue. Hence, an HA-tagged AUP1 construct, in which all ten lysine residues were mutated to arginines (AUP1-10KR-HA) was generated and analyzed with respect to its ubiquitination pattern. Ubiquitination was

undetectable for AUP1-10KR-HA (Fig. 15, B). Together, these results demonstrate that AUP1 is ubiquitinated on several lysine residues and that multiple K-to-R changes are required for the inhibition of AUP1 ubiquitination.

Next, we wanted to know if any of the lysine to arginine mutations has an effect on LD clustering. Overexpression of the single AUP1 K-to-R mutants in COS-7 cells caused clustering of LDs to comparable extends as COS-7 cells overexpressing AUP1-HA (Fig. 15, C). In contrast, LD clustering was strongly attenuated in COS-7 cells overexpressing AUP1-10KR-HA (Fig. 15, C and Fig. 16) and was similar to COS-7 cells overexpressing AUP1 with a deleted or mutated CUE domain, although AUP1-10KR-HA retained the CUE domain. These results suggest that the AUP1 CUE domain alone is not sufficient to promote LD clustering. Rather, the CUE domain is necessary for AUP1 ubiquitination, which in turn is necessary to promote LD clustering.

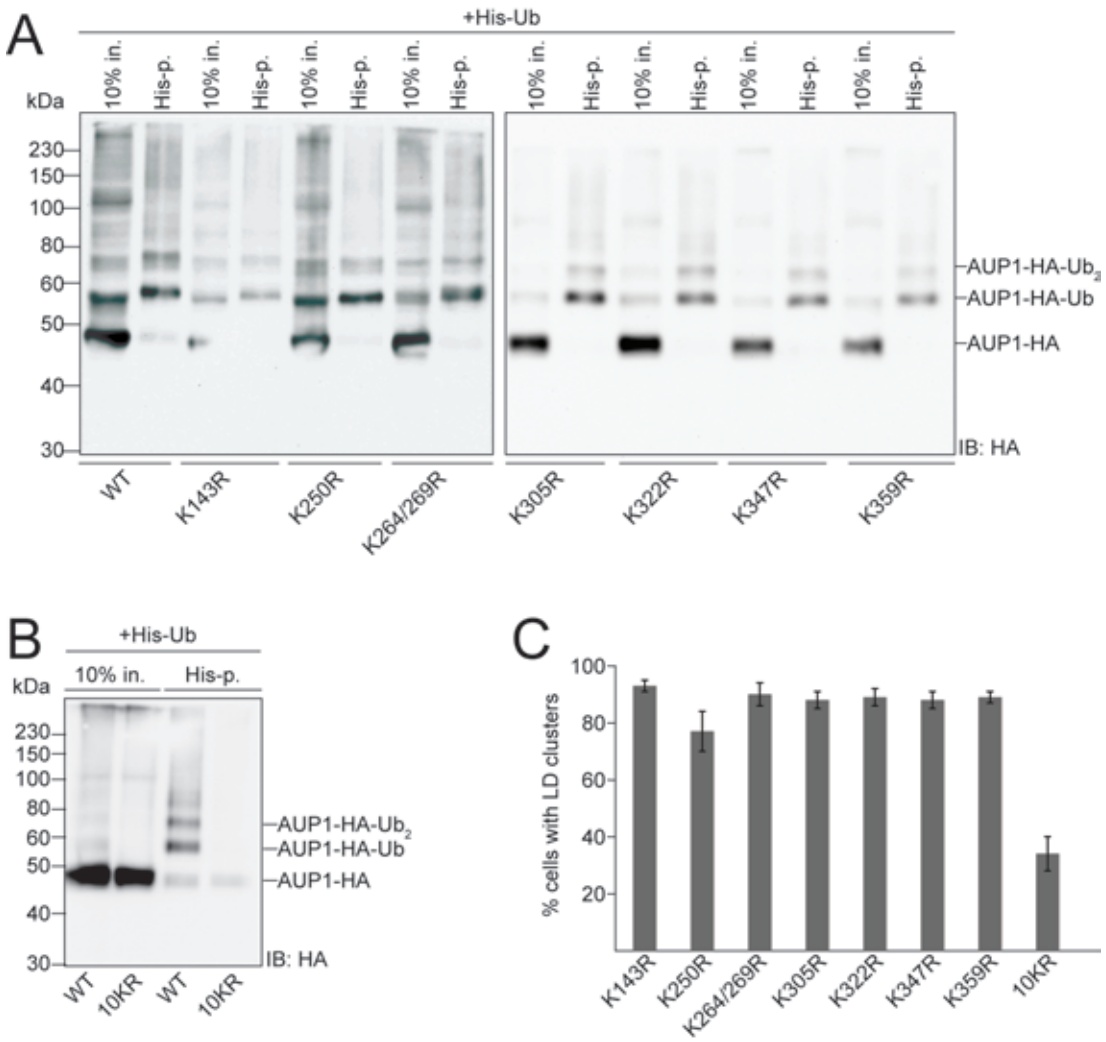

Figure 15.

AUP1 is ubiquitinated at multiple lysine residues

(A) COS-7 cells were transfected with His-ubiquitin and HA-tagged AUP1 constructs (as indicated). His-ubiquitin and His-ubiquitin modified proteins were purified over Ni-NTA agarose. Proteins from lysates and His-purifications were separated on SDS-PAGE and immunoblotted with anti-HA antibody. (B) COS-7 cells were transfected with His-ubiquitin and HA-tagged AUP1 constructs (as indicated). Samples were processed as under (A). (C) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed.

Imortantly, neither the single K-to-R mutations (not shown) nor the mutation of all ten lysine residues to arginines changed the localization of AUP1 to LDs (Fig. 16, inset).

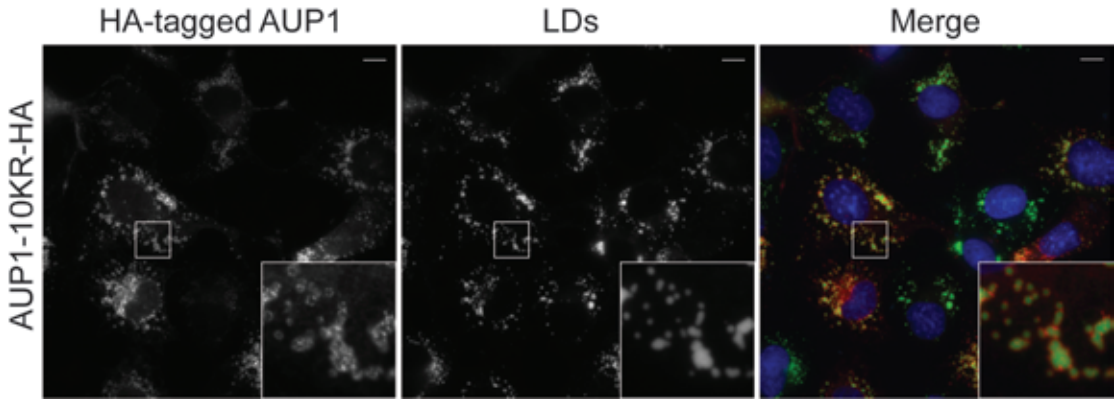

Figure 16.

Non-ubiquitinated AUP1 fails to induce LD clustering

Fluorescence micrographs of COS-7 cells overexpressing AUP1-10KR-HA and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panel), LDs were stained with LD540 (middle panel). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panel). Bars, 10μm.

4.7. AUP1 monoubiquitination is sufficient to promote LD clustering

In order to address the question whether AUP1 ubiquitination is sufficient to promote LD clustering, even in absence of a functional CUE domain, we constructed HA-tagged chimeras of AUP1-ΔCUE (AUP1-ΔCUE-UbK48R-HA) and AUP1-mutCUE2 (AUP1-mutCUE2-UbK48R-HA) fused to ubiquitin. Lys48 on ubiquitin was further mutated to arginine (UbK48R) to prevent further polyubiquitination of the chimera proteins. Overexpression of these chimera proteins in COS-7 cells induced LD clustering to comparable extends as AUP1-HA, with around 90% of cells showing LD clustering (Fig. 17, A, B and D). Also, overexpression of HA-tagged UbK48R fused to AUP1-10KR (AUP1-10KR-UbK48R-HA) induced LD clustering, with around 90% of COS-7 cells showing LD clustering (Fig. 17, C and D).

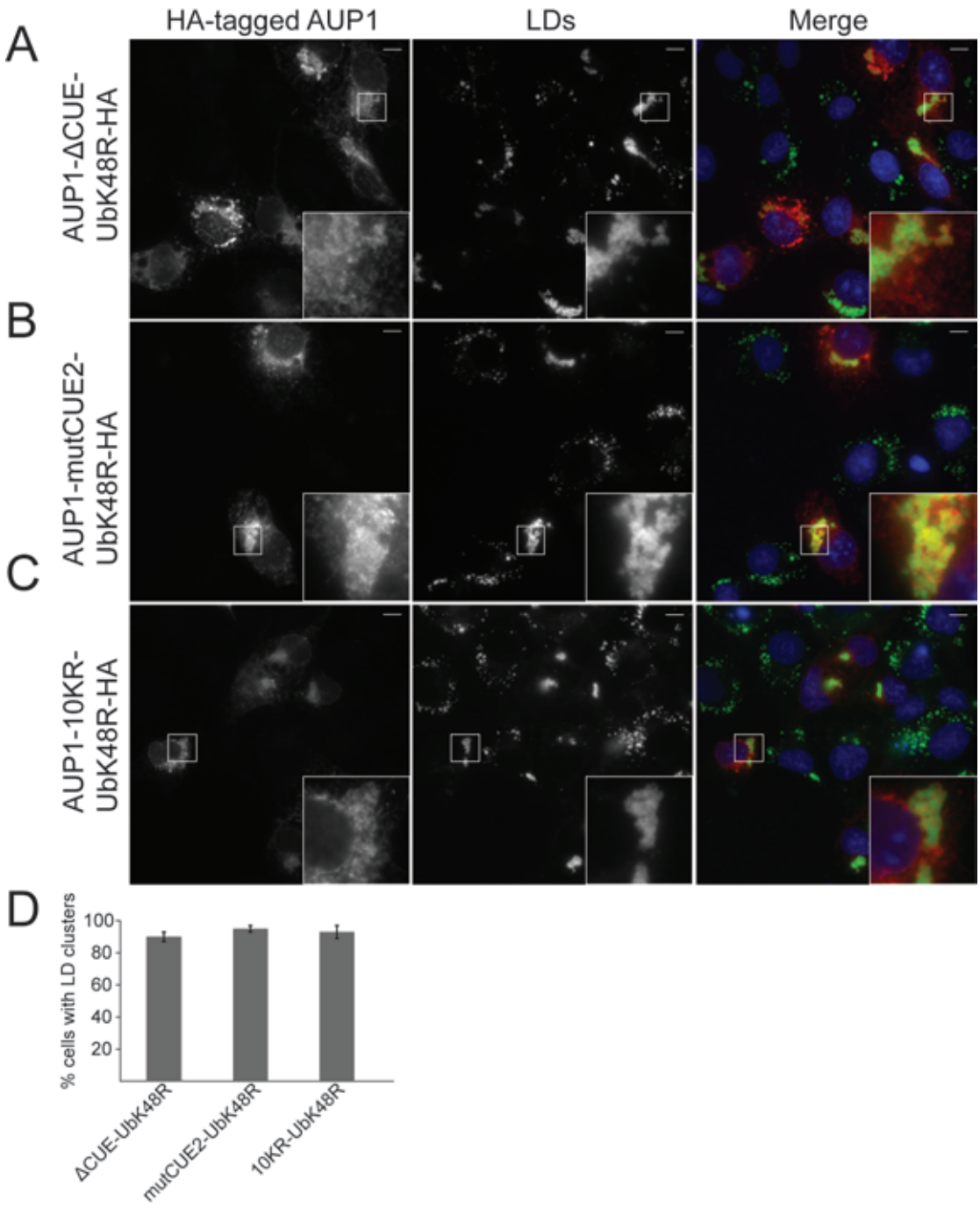

Figure 17.

AUP1 monoubiquitination is sufficient to promote LD clustering

(A-C) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated) and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panels), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm. (D) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed.

From the above data we conclude that the addition of a single ubiquitin moiety to AUP1 is sufficient to promote LD clustering and that this process is independent of the CUE domain. Yet, the chimera proteins did not form the ring-like structures around individual LDs (Fig. 17, A-C, inset) as was observed for AUP1-HA (compare to fig. 11, A, inset). However, the AUP1 ubiquitin chimera proteins are enriched at the sites of LD clusters (Fig. 17, A-C, inset) (see discussion).

Attention should be paid to the fact that the observed LD clustering seemed to be independent of the expression levels of the used constructs. LD clustering was also induced in cells having very low expression levels of the chimera proteins. Figure 18 shows COS-7 cells overexpressing AUP1-ΔCUE-UbK48R-HA and an additional merged image with overexposed signal from the chimera protein. Even cells with very low expression levels showed LD clustering whereas only cells without any detectable expression of the fusion protein showed no LD clustering.

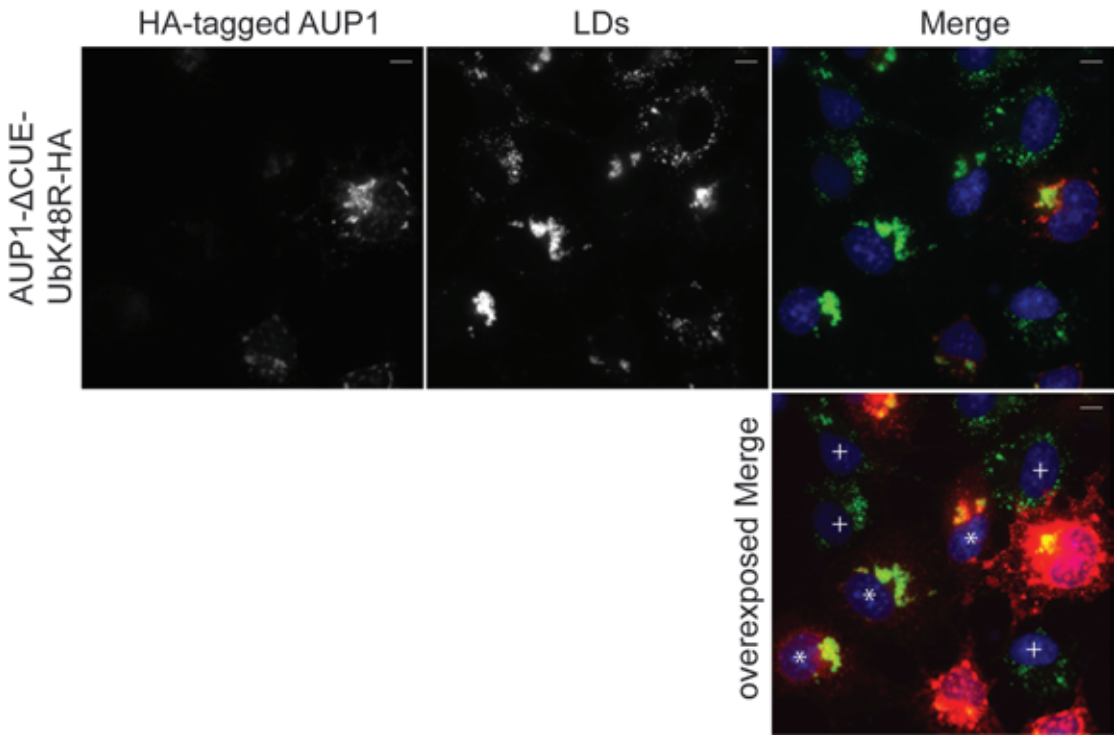

Figure 18.

Low expression levels of the AUP1 ubiquitin chimera proteins are sufficient to induce LD clustering

Fluorescence micrographs of COS-7 cells overexpressing AUP1-ΔCUE-UbK48R-HA and supplemented with 50 µM oleate are shown. Cells were immunostained with anti-HA antibody (left panel), LDs were stained with LD540 (middle panel). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panel). Bars, 10µm. The overexposed Merge shows LD clustering only for cells overexpressing the chimera protein (marked with asterisk (*)), cells without detectable expression show no LD clustering (marked with plus (+)).

The ubiquitin fused to AUP1 might be targeted for polyubiquitin chain assembly. Although Lys48 has been mutated to arginine, a different lysine on ubiquitin might be targeted for assembly of a polyubiquitin chain. The His-tag affinity purification was therefore used to analyze whether AUP1-mutCUE2-UbK48R-HA is further ubiquitinated. Full length AUP1 fused to HA-tagged UbK48R (AUP1-UbK48R-HA) was used as a control to analyze whether further ubiquitination is possible if the CUE domain is retained within the chimera protein. AUP1-mutCUE2-UbK48R-HA and AUP-WT-UbK48R-HA exhibited strongly reduced expression levels compared to AUP1-HA (Fig. 19). AUP1-mutCUE2-UbK48R-HA and AUP-UbK48R-HA

migrated at approximately the same molecular weight as monoubiquitinated AUP1-HA. Also, AUP1-WT-UbK48R-HA is further modified by one ubiquitin moiety while AUP1-mutCUE2-UbK48R-HA lacked any further ubiquitination (Fig. 19). Although expressed at much lower levels than AUP1-HA, the AUP1-mutCUE2-UbK48R-HA construct appeared to be stable and not further ubiquitinated, validating that a single ubiquitin moiety fused to AUP1 is sufficient to promote LD clustering.

In addition, a specific band appeared for both AUP1 ubiquitin chimera proteins, migrating at an apparent molecular weight of around 100kDa (see 4.10. AUP1 forms stable dimers).

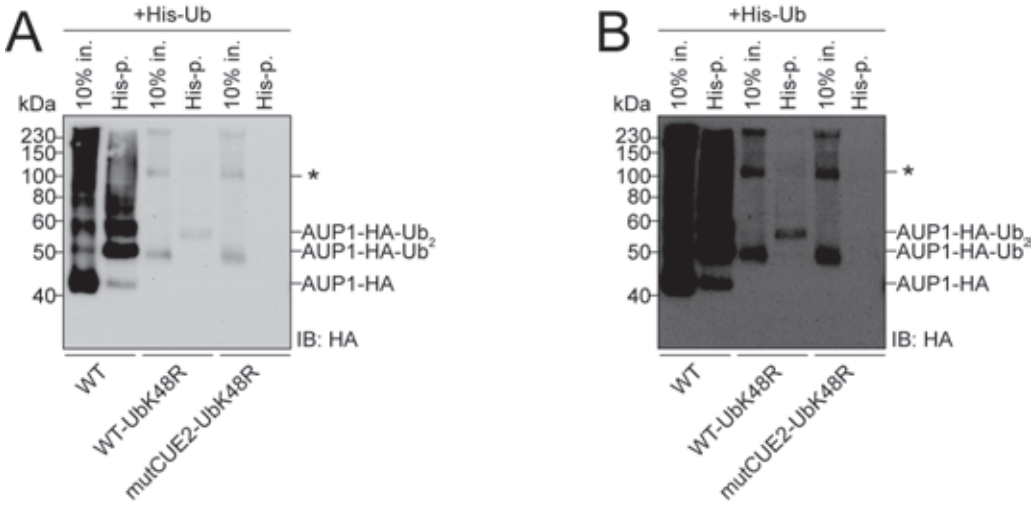

Figure 19.

Western blot analysis of AUP1 ubiquitin chimera proteins

COS-7 cells were transfected with His-ubiquitin and HA-tagged AUP1 constructs (as indicated). His-ubiquitin and His-ubiquitin modified proteins were purified from cell lysates over Ni-NTA agarose. Proteins from lysates (10% input) and His-purifications were separated on SDS-PAGE and immunoblotted with anti-HA antibody. Note: a specific band appears for both AUP1 ubiquitin fusion proteins migrating at an apparent molecular weight of around 100kDa (marked with asterisk (*)). (B) Overexposed signal from (A).

4.8. Conserved residues on ubiquitin not required for LD clustering

Monoubiquitinated proteins are often recognized by other UBD containing proteins, which in turn function in downstream signaling events. Such interactions might be important for monoubiquitinated AUP1-induced LD clustering. Amino acid residues on ubiquitin known to mediate interaction with UBDs are isoleucine 44 (I44) and aspartic acid 58 (D58). We therefore generated two AUP1-ΔCUE constructs, each fused to HA-tagged UbK48R carrying itself an additional I44A or D58A mutation (AUP1-ΔCUE-UbI44A-K48R-HA and AUP1-ΔCUE-UbK48R-D58A-HA).
Overexpression of either construct in COS-7 cells still caused the clustering of LDs (Fig. 20, A and B).

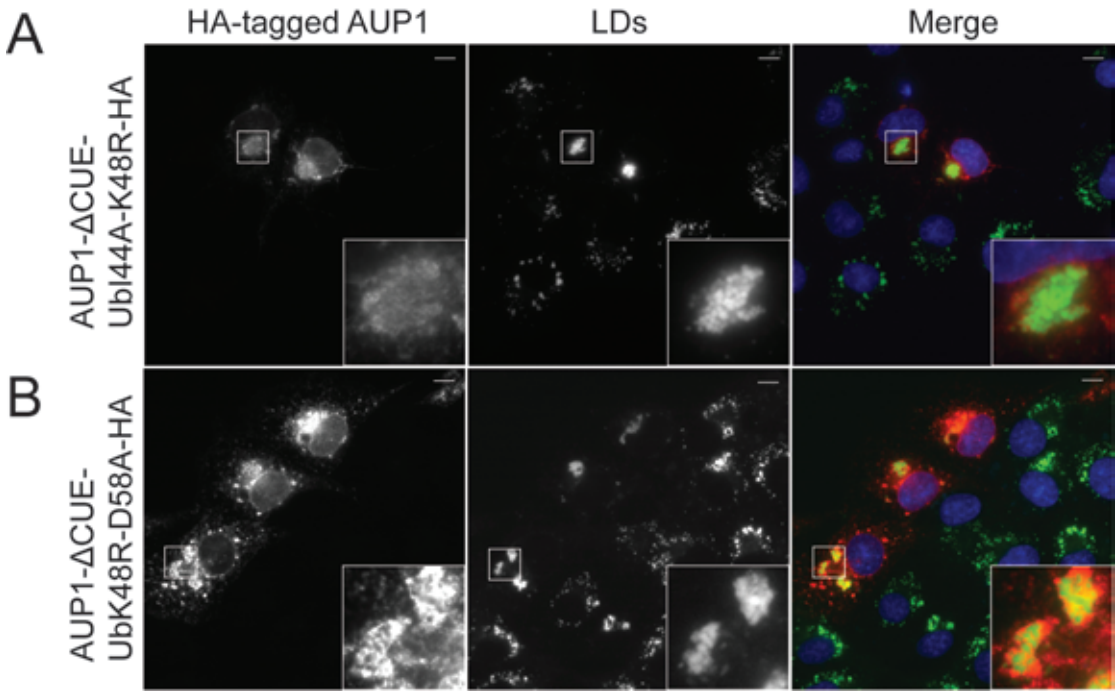

Figure 20.

Conserved residues on ubiquitin are not required for LD clustering

(A-B) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated) and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panels), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm.

These results suggest that conserved residues isoleucine 44 and aspartic acid 58 on ubiquitin are not required for LD clustering induced by the ubiquitination of AUP1.

4.9. AUP1 localization to LDs is necessary for LD clustering

It was shown that AUP1 is ubiquitinated in a CUE dependent fashion and that this ubiquitination is necessary to promote LD clustering. Also, irrespective of the changes introduced into AUP1, each construct still localized to LDs. Only for the AUP1 ubiquitin chimera proteins localization to LDs was not as readily observed. It was therefore analyzed whether the localization of AUP1 to LDs is a prerequisite to induce their clustering.

Like several other integral LD proteins, AUP1 exhibits a dual distribution in cells, localizing to LDs and the ER. In collaboration with Ana Stevanovic, LD clustering was analyzed in COS-7 cells overexpressing HA-tagged AUP1 mutants (AUP1-mutLDT1-HA and AUP1-mutLDT2-HA) that localize only to the ER and fail to reach LDs (Stevanovic and Thiele, 2012). Figure 21, A shows COS-7 cells overexpressing AUP1-mutLDT1-HA, which localized to the ER but not to LDs. LD clustering could not be observed in COS-7 cells overexpressing either, AUP1-mutLDT1-HA or AUP1-mutLDT2-HA, similar to COS-7 cells transfected with an empty control vector (Fig. 21, B). These results suggest that AUP1 localization to LDs is necessary to promote LD clustering.

However, AUP1 localization to LDs might be a prerequisite for its ubiquitination, which in turn is necessary for LD clustering. The His-tag affinity purification was used to analyze whether the AUP1-mutLDT mutants are still ubiquitinated. As shown in Figure 21, C AUP1-mutLDT1-HA and AUP1-mutLDT2-HA exhibited the same ubiquitination pattern as AUP1-HA, suggesting that AUP1 localization to LDs is not necessary for AUP1 ubiquitination. Taken together, these results demonstrate that AUP1 localization to LDs is necessary to promote LD clustering.

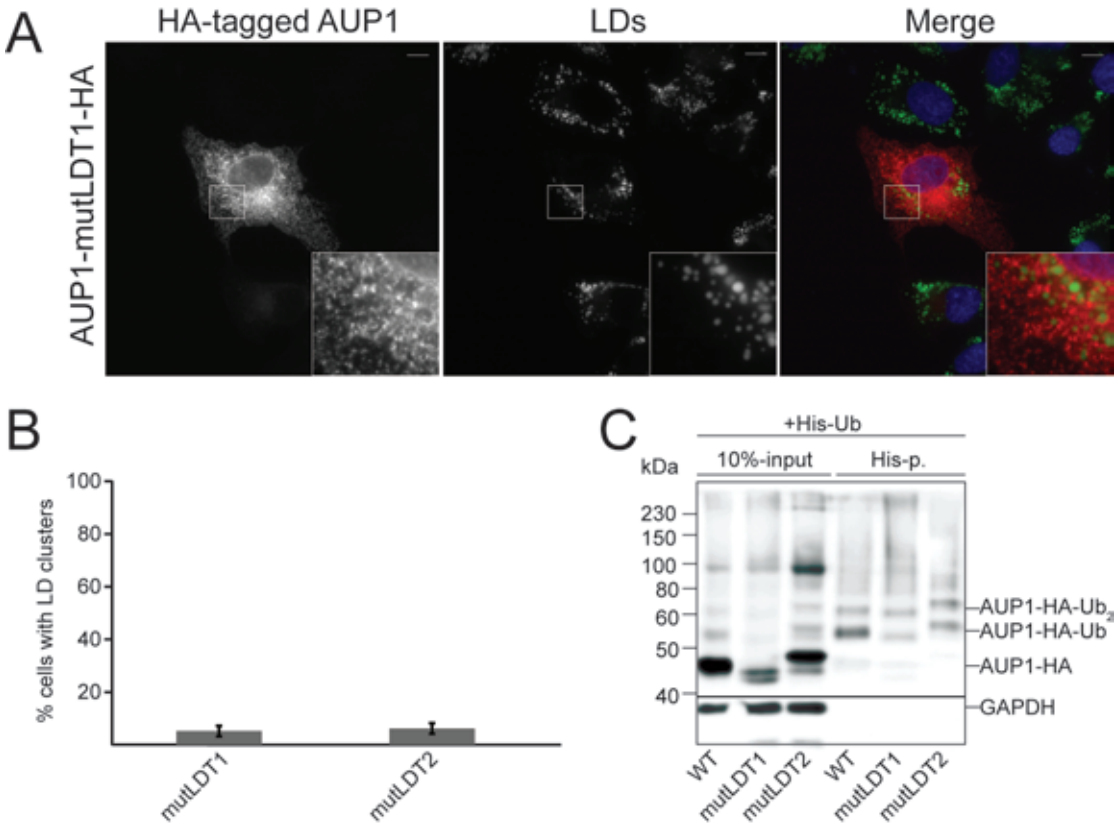

Figure 21.

AUP1 localization to LDs is necessary for LD clustering

(A) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1-mutLDT1 and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panel), LDs were stained with LD540 (middle panel). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panel). Bars, 10μm. (B) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed. Data for (B) taken from PhD thesis, Stevanovic Ana (2012). (C) COS-7 cells were transfected with His-ubiquitin and HA-tagged AUP1 constructs (as indicated). His-ubiquitin and His-ubiquitin modified proteins were purified over Ni-NTA agarose. Proteins from lysates and His-purifications were separated on SDS-PAGE and immunoblotted with anti-HA antibody. GAPDH served as loading control.

4.10. AUP1 forms stable dimers

While working with AUP1, we repeatedly observed a high molecular weight band for AUP1 on western blots migrating at an apparent molecular weight of around 100 kDa, twice the molecular weight of AUP1 (for examples see fig. 14). The observed band at around 100 kDa was especially prominent for the overexpressed AUP1 ubiquitin chimera proteins (Fig. 19). Dimerization of AUP1 could represent a possible mean to drive LD clustering (see discussion). It was therefore speculated whether the observed band might be an AUP1 dimer. To analyze possible AUP1 dimerization, AUP1 self-interaction was studied by a pull-down approach.

Whole cell extract from COS-7 cells overexpressing myc- and HA-tagged AUP1 were immunoprecipitated with a myc-tag specific antibody. HA-tagged AUP1 was efficiently co-purified with myc-tagged AUP1 while HA-tagged AUP1 was not co-purified in the absence of myc-tagged AUP1 (Fig. 22, A). As expected, myc-tagged AUP1 was efficiently pulled down by the myc-tag specific antibody (Fig. 22, B). To exclude the possibility that we unspecifically pulled down LD proteins, the immunoprecipitate was also analyzed for contamination with the LD proteins NSDHL and ACSL3 (acyl-CoA synthetase long-chain family member3) . Both proteins were present in the cell lysate but were absent in the immunoprecipitate (Fig. 22, C). Also, the observed band at the molecular weight of around 100 kDa appeared in the immunoprecipitate and was positive for both, HA- and myc-tagged AUP1.

Next, we examined myc-tagged AUP1 mutants for their ability to pull-down AUP1-HA. We used AUP1-ΔCUE-myc and an AUP1 construct with point mutations within the G2BR domain (AUP1-mutG2BR-myc) to analyze whether any of the two C-terminally located domains are important for AUP1 dimerization. Both, AUP1-ΔCUE-myc and AUP1-mutG2BR-myc efficiently pulled down AUP1-HA. Again, both myc-tagged constructs were present in the immunoprecipitate while NSDHL and ACSL3 were not co-purified (Fig., 22, A, B and C). These results suggest that AUP1 can form stable dimers and that neither, a functional G2BR nor CUE domain on one interacting AUP1 partner is necessary for this process. In addition, since the CUE domain is necessary for AUP1 ubiquitination, these results suggest that AUP1 ubiquitination is not required for the dimerization of AUP1.

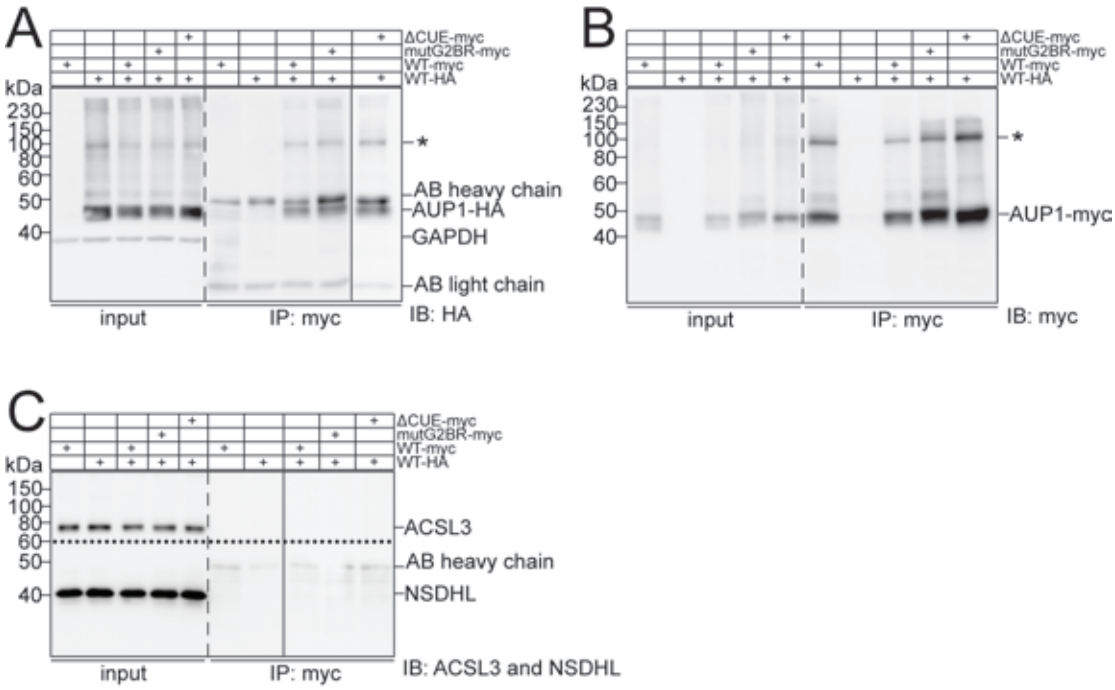

Figure 22.

AUP1 forms stable dimers

COS-7 cells were transfected with HA-tagged AUP1 (WT) and myc-tagged AUP1 constructs (as indicated). Myc-tagged and associated proteins were immunoprecipitated with myc-tag specific antibody and separated on SDS-PAGE and immunoblotted with (A) anti-HA antibody, (B) anti-myc antibody or (C) membrane was cut in half (indicated by dotted line). Upper part was immunoblotted with anti-ACSL3 and lower part with anti-NSDHL antibody. Putative AUP1 dimers are marked with asterisk (*).

4.11. Phosphorylation of AUP1

AUP1 is a highly phosphorylated protein (Dephoure *et al.*, 2008; Kim *et al.*, 2006; Yang *et al.*, 2006). A function to these posttranslational modifications has only been suggested for phosphorylation of Thr68. Preliminary data suggested that exchanging AUP1 Thr68 by the negatively charged aspartic acid, thereby mimicking the phosphorylated state of Thr68, negatively interferes with LD clustering. In contrast, exchanging AUP1 Thr68 for alanine, thereby mimicking the non-phosphorylated state of Thr68 does not change the clustering of LDs (Spandl Johanna, PhD thesis 2009). The same HA-tagged AUP1 constructs (AUP1-T68D-HA and AUP1-T68A-HA) were

used to further investigate and quantify the effect of the assumed phosphorylation of AUP1 Thr68 on LD clustering.

Overexpression of AUP1-T68D-HA in COS-7 cells caused LD clustering in only around 20% of cells, whereas COS-7 cells overexpressing AUP1-T68A-HA showed LD clustering to a comparable degree than AUP1-HA overexpressing COS-7 cells (Fig. 23, A and B). These results indicate that phosphorylation of AUP1 at Thr68 negatively interferes with the ability of AUP1 to promote LD clustering. However, it has to be noted that expression levels of AUP1-T68D-HA were very low compared to AUP-HA or AUP1-T68A-HA (see below). Importantly, exchanging Thr68 for aspartic acid or alanine did not change the localization of AUP1 to LDs (Fig. 23, A, inset).

Phosphorylation is a wide spread posttranslational modification with diverse functions. For several proteins it was shown that phosphorylation influences ubiquitination of the phosphorylated protein (Hunter, 2007). Results shown so far demonstrated the importance of AUP1 ubiquitination for the induction of LD clustering. Phosphorylation of AUP1 might therefore control LD clustering by negatively influencing the ubiquitination state of AUP1. Hence, the His-tag affinity purification was used to analyze whether the AUP1-T68D mutation influences AUP1 ubiquitination. Yet, AUP1-T68D-HA and AUP1-T68A-HA exhibited the same ubiquitination pattern as AUP1-HA (Fig. 23, C), suggesting that the phosphorylation of AUP1 Thr68 does not attenuate LD clustering by obviating AUP1 ubiquitination.

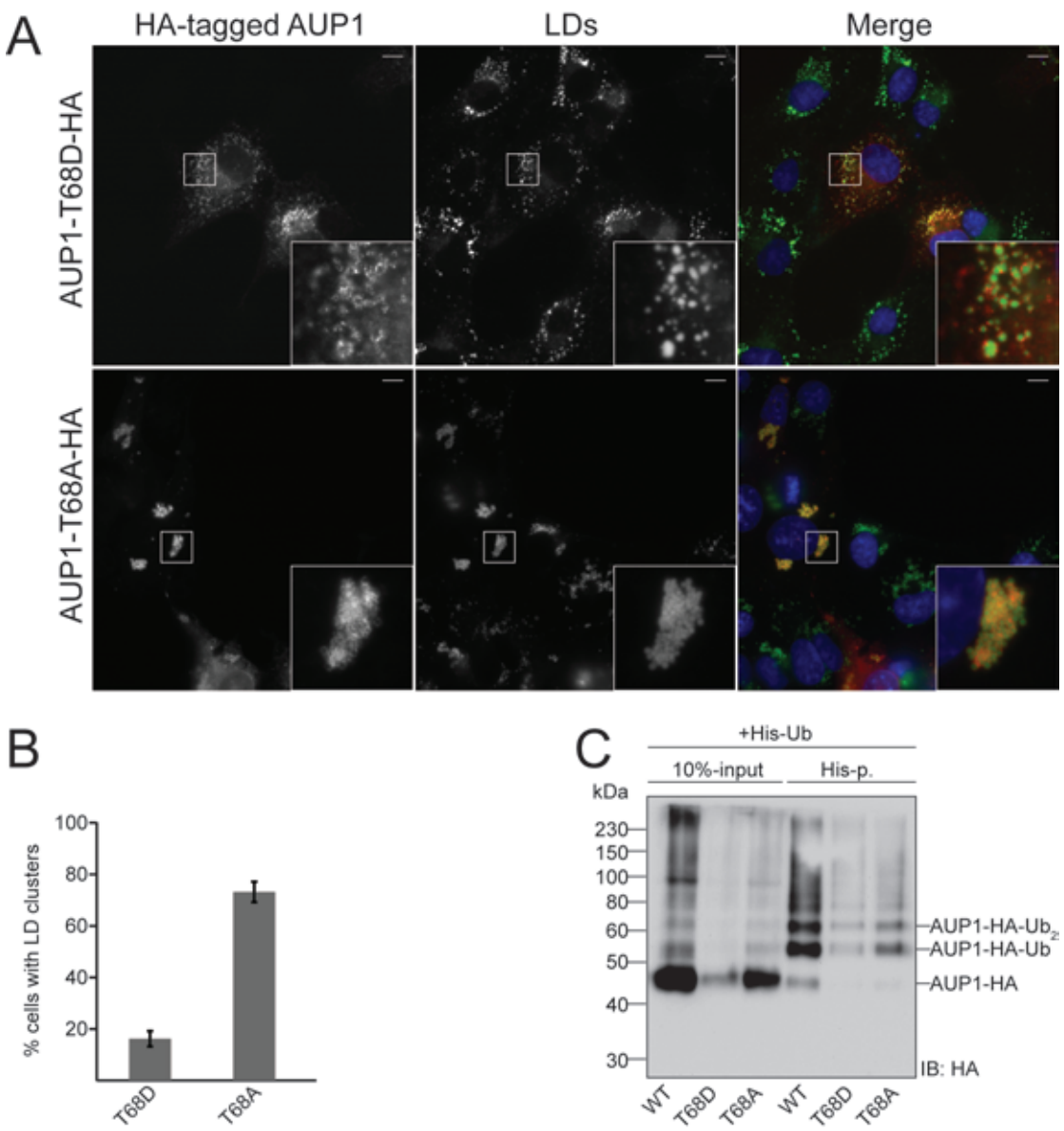

Figure 23.

Mimicking phosphorylation of AUP1 Thr68 prevents LD clustering

(A) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated) and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panel), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm. (B) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed. (C) COS-7 cells were transfected with His-ubiquitin and HA-tagged AUP1 constructs (as indicated). His-ubiquitin and His-ubiquitin modified proteins were purified over Ni-NTA agarose. Proteins from lysates and His-purifications were separated on SDS-PAGE and immunoblotted with anti-HA antibody

As apparent from Figure 23, C, expression levels of AUP1-T68D-HA were strongly reduced compared to AUP1-HA or AUP1-T68A-HA. The possibility therefore remained that decreased expression levels of AUP1-T68D-HA attenuated the clustering of LDs. As shown before, AUP1-ΔG2BR-HA had much higher expression levels than AUP1-HA and caused LD clustering to comparable extends as AUP1-HA (Fig. 12, A and B). To increase stability of AUP1-T68D-HA, the T68D mutation was introduced into HA-tagged AUP1-ΔG2BR (AUP1-T68D-ΔG2BR-HA), which led to strongly elevated expression levels compared to AUP1-T68D-HA alone. HA-tagged AUP1-T68A-ΔG2BR was cloned as a control to exclude the possibility that deletion of the G2BR domain in the context of a mutated Thr68 residue has an influence on LD clustering. Again, AUP1-T68D-ΔG2BR-HA overexpressed in COS-7 cells (Fig. 24, A) caused LD clustering in only around 20% of cells (Fig. 24, B) whereas around 80% of COS-7 cells overexpressing AUP1-T68A-ΔG2BR-HA showed LD clustering (Fig. 24, A and B). These results suggest that the reduced expression levels of AUP1-T68D-HA are not responsible for the observed change in LD clustering. Taken together, these results indicate that phosphorylation of AUP1 Thr68 prevents LD clustering or actively promotes LD declustering.

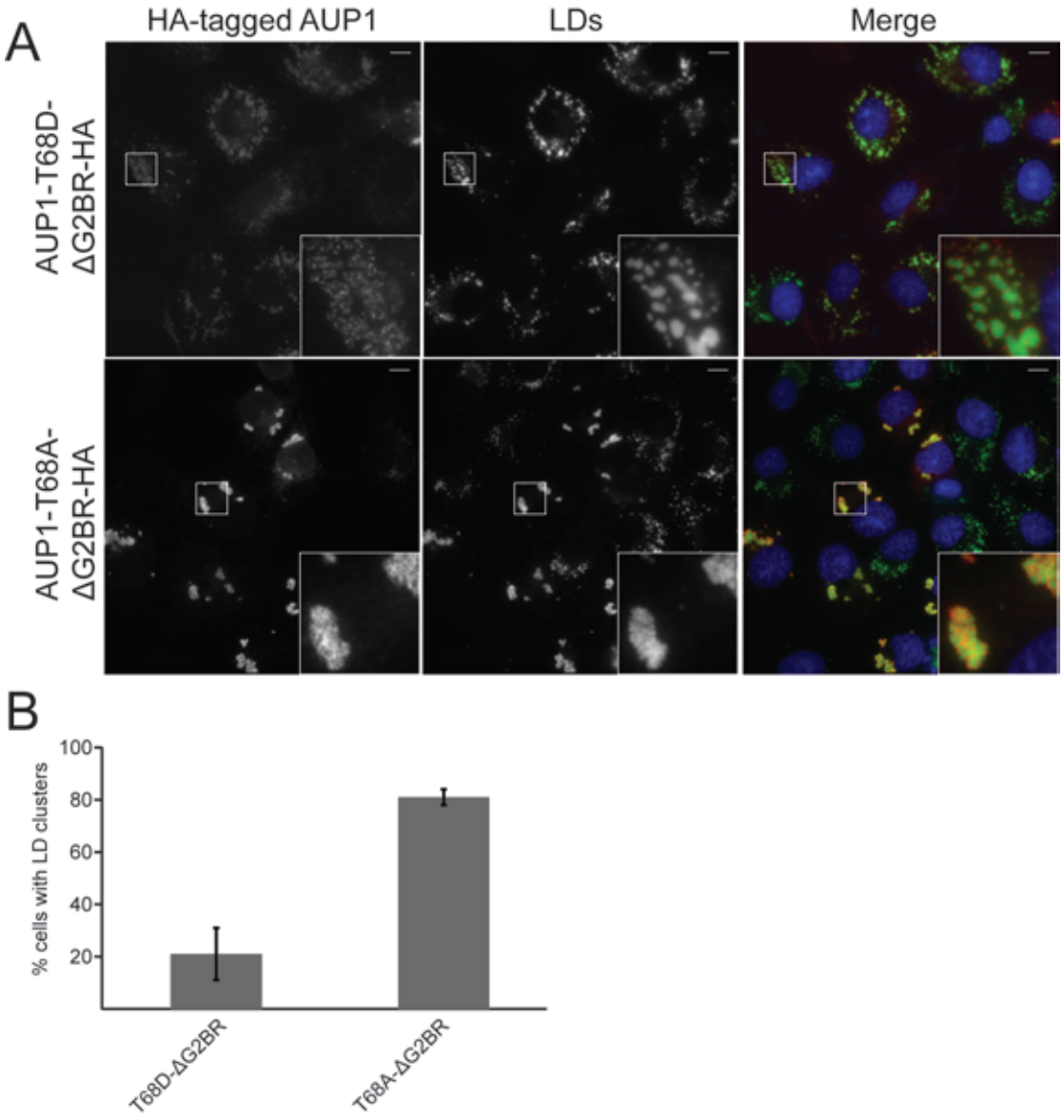

Figure 24.

Reduced expression levels of AUP1 phosphomimetic mutants are not responsible for reduced clustering of LDs

(A) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated) and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panels), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm. (B) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed.

4.12. AUP1-T68D-ΔCUE, abolishing LD clustering

So far, several mutations were found to attenuate the ability of AUP1 to promote LD clustering. Yet, preventing ubiquitination of AUP1 or mimicking phosphorylation of AUP1 at residue Thr68 did not decrease LD clustering to the same level as in cells transfected with an empty control vector. Possibly, preventing the ubiquitination of AUP1-T68D-HA could further decrease LD clustering. It was therefore analyzed whether AUP1-ΔCUE combined with the T68D mutation would further attenuate the clustering of LDs.

Indeed, COS-7 cells overexpressing AUP1-T68D-ΔCUE-HA showed LD clustering to a lesser extent than COS-7 cells overexpressing AUP1-T68D-HA or AUP1-ΔCUE-HA, and to a similar extend as COS-7 cells transfected with an empty control vector (Fig. 25, A and B). On the other hand, COS-7 cells overexpressing AUP1-T68A-ΔCUE-HA showed LD clustering to a similar degree as COS-7 cells overexpressing AUP1-ΔCUE-HA alone (Fig. 24, A and B). Again, exchanging Thr68 for aspartic acid or alanine in an AUP1-ΔCUE background did not change the localization of AUP1 to LDs (Fig. 25, A, inset). These results suggest that Thr68 phosphorylated AUP1 still retains a partial ability to promote LD clustering due to the prevailing ubiquitination.

Taken together, results presented here suggest that posttranslational modifications of AUP1 through ubiquitination and phosphorylation antagonistically control LD clustering.

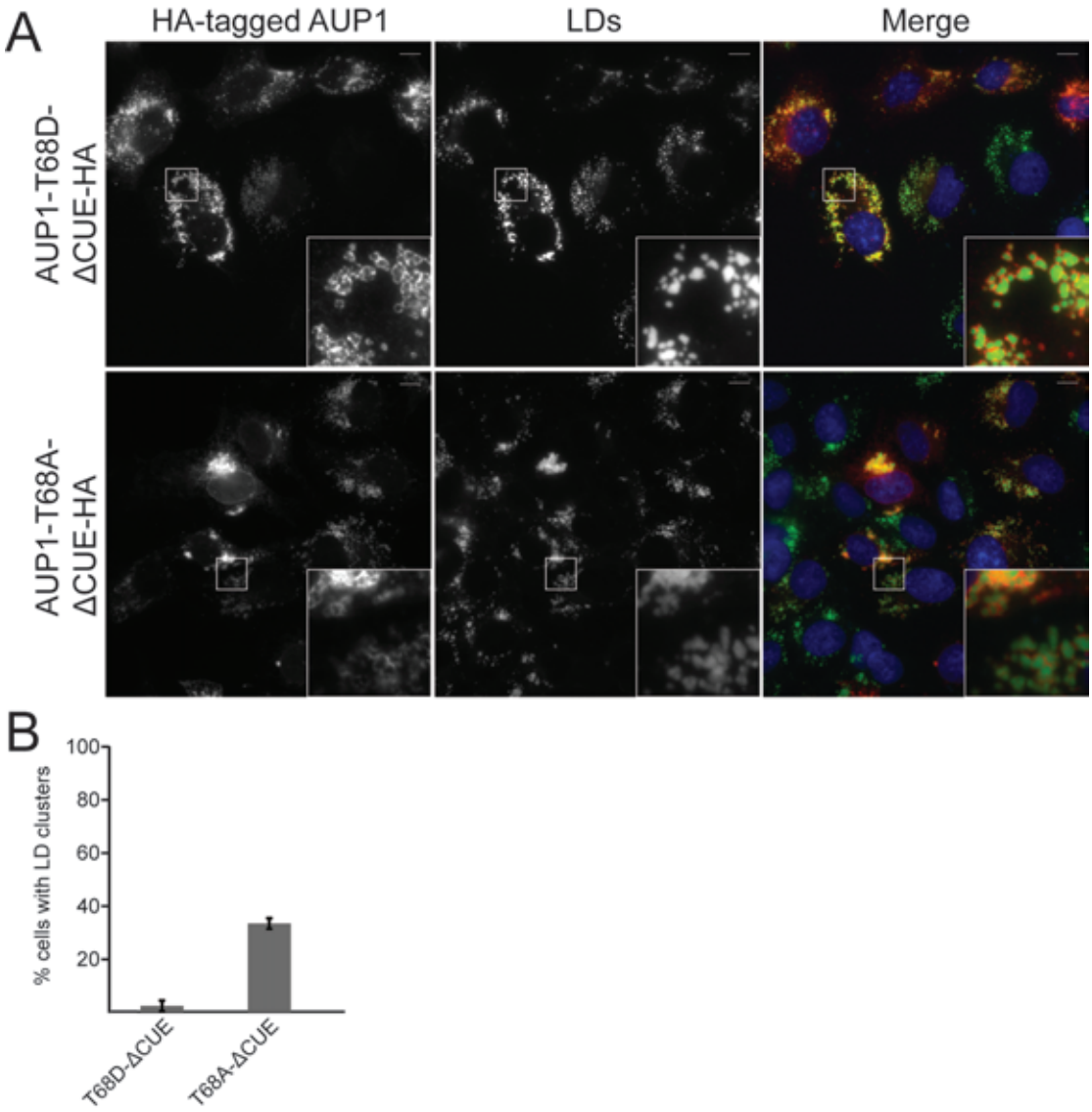

Figure 25.

LD clustering is abolished by phosphorylation of AUP1 lacking ubiquitination

(A) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated) and supplemented with 50 μM oleate are shown. Cells were immunostained with anti-HA antibody (left panels), LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, AUP1 in red and LDs in green are shown (right panels). Bars, 10μm. (B) Quantification of LD clustering in COS-7 cells overexpressing HA-tagged AUP1 constructs (as indicated). Results are average of three independent experiments with standard deviation. For each individual experiment at least 25 cells were analyzed.

4.13. AUP1 T68 phosphorylation does not prevent AUP1 dimerization

Mimicking AUP1 phosphorylation at Thr68 did not influence AUP1 ubiquitination, but might have an influence on AUP1 dimerization. Dimerization of myc-tagged AUP1 with AUP1-T68D-ΔG2BR-HA or AUP1-T68A-ΔG2BR-HA was hence analyzed by pull-down approach. The AUP1-T68D-HA mutant could not be used for the pull-down experiment, the low expression levels obviated a reliable detection after immunoprecipitation.

Whole cell extract from COS-7 cells overexpressing myc-tagged AUP1 and HA-tagged AUP1-T68D-ΔG2BR or AUP1-T68A-ΔG2BR were immunoprecipitated with myc-tag specific antibody. HA-tagged AUP1 constructs were efficiently co-purified with myc-tagged AUP1 (Fig. 26, A). Again, myc-tagged AUP1 was efficiently pulled down by the myc-tag specific antibody (Fig. 26, B). The LD proteins NSDHL and ACSL3 were present in the cell lysate but were absent from the immunoprecipitate (Fig. 26, B).

AUP1 ubiquitination and phosphorylation might both control AUP1 dimerization independent from each other, and possibly AUP1 dimerization is inhibited by an AUP1-T68D mutant lacking ubiquitination. Dimerization of myc-tagged AUP1 with HA-tagged AUP1-T68D-ΔCUE and AUP1-T68A-ΔCUE was therefore analyzed by pull-down approach. Again, HA-tagged AUP1 constructs were efficiently co-purified with myc-tagged AUP1 (Fig. 26, A). Also, myc-tagged AUP1 was efficiently pulled down by the myc-tag specific antibody (Fig. 26, B) and the LD proteins NSDHL and ACSL3 were present in the cell lysate but were absent from the immunoprecipitate (Fig. 26, B). Taken together these results suggest that neither the phosphorylation of AUP1 at Thr68 nor the ubiquitination of AUP1 are required for AUP1 dimerization.

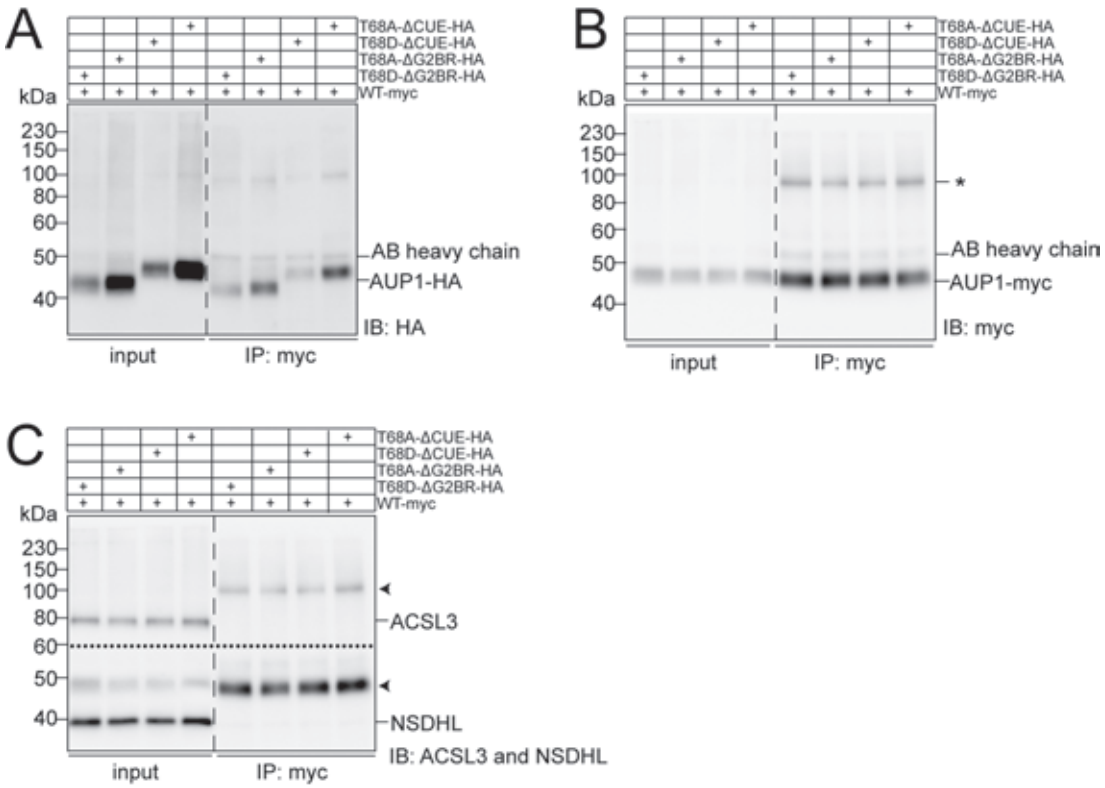

Figure 26.

AUP1 phosphomimetic mutants form stable dimers

COS-7 cells were transfected with HA-tagged AUP1 and myc-tagged AUP1 constructs (as indicated). Myc-tagged and associated proteins were immunoprecipitated with myc-tag specific antibody and separated on SDS-PAGE and immunoblotted with (A) anti-HA antibody and (B) anti-myc antibody. (C) Antibodies from membrane used for (B) were stripped and membrane was cut in half (indicated by dotted line). Upper part was immunoblotted with anti-ACSL3 and lower part with anti-NSDHL antibody. Note: Residual signal from myc-tagged AUP1 is still visible (indicated by arrowheads). Putative AUP1 dimers are marked with asterisk (*).

5. Discussion

5.1. LD clustering

Over the last decades LDs have been intensively studied and many aspects concerning the formation, growth and regulation of LDs have been addressed by numerous studies. The work of this thesis focused on an aspect of LD dynamics described only by few studies so far, the clustering of LDs. LDs have been observed to aggregate and form clusters in different organisms like yeast (Binns *et al.*, 2006), *Drosophila* (Zhang *et al.*, 2000; Scott *et al.*, 2004) or *Arabidopsis thaliana* (Kuang and Musgrave, 1996) and several cell types ranging from *Drosophila* Schneider2 cells (Guo *et al.*, 2008) to adipocytes (Boström *et al.*, 2005; Wolins *et al.*, 2005) and hepatocytes (Fowler and Greenspan, 1985). Also, several LD associated proteins have been described to promote LD clustering upon overexpression in cells, as is the case for caveolin-1 (Ostermeyer *et al.*, 2001), core protein of HCV (Boulant *et al.*, 2008; Depla *et al.*, 2010), fat specific protein27 (fsp27) (Jambunathan *et al.*, 2011), methyltransferase like 7b (MettL7b) (Turró *et al.*, 2006), perilipin A (Marcinkiewicz *et al.*, 2006; Miura *et al.*, 2002) and perilipin5 (Granneman *et al.*, 2009). Yet, for most proteins any mechanistic insight into how LD clustering is achieved is lacking so far. However, the localization to LDs seems to be a prerequisite to promote LD clustering, since failure in localizing to LDs of fsp27 (Jambunathan *et al.*, 2011) and core protein of HCV (Hourioux *et al.*, 2007; Boulant *et al.*, 2008) was shown to also obviate their ability to promote LD clustering.

AUP1 was identified in our lab as an LD associated protein, and results from this thesis demonstrate that overexpression of AUP1 in COS-7 cells caused LD clustering in a quantitative manner.

5.2. AUP1 and its connection to LD clustering

AUP1 is a ubiquitously expressed protein and the A431 cell line was found to have relatively high expression levels of AUP1. Results from this thesis demonstrate that knock-down of AUP1 in A431 cells quantitatively reduced the otherwise predominant clustering of LDs observed in this cell line. Thus indicating that AUP1 is important for the clustering of LDs in this cell line and that endogenous expression levels of AUP1 can be sufficient to promote LD clustering. Yet, knock-down of AUP1 in A431 cells did not inhibit LD clustering in all cells analyzed, suggesting that residual AUP1 expression might be enough to promote LD clustering in some cells. In addition, it cannot be excluded that other mechanisms in A431 cells exist which might promote clustering of LDs, independent of AUP1. A complete knock-out of AUP1 in the A431 cell line would show whether complete loss of endogenous AUP1 expression would be sufficient to entirely obviate LD clustering in this cell line (see 5.10.).

In order to dissect the molecular mechanism behind AUP1-induced LD clustering, several AUP1 mutants were generated and analyzed for their ability to promote LD clustering in COS-7 cells. An overview of the AUP1 constructs used in this thesis and analyzed with respect to their ubiquitination state, ability to induce LD clustering, integrity of the CUE domain and localization to LDs is given in table 3.

Construct	Ubiquitinated	LD clustering	CUE domain	LD localization
K143R	Yes	Yes	Yes	Yes
K250R	Yes	Yes	Yes	Yes
K264/269R	Yes	Yes	Yes	Yes
K305R	Yes	Yes	Yes	Yes
K322R	Yes	Yes	Yes	Yes
K347R	Yes	Yes	Yes	Yes
K359R	Yes	Yes	Yes	Yes
ΔG2BR	Yes	Yes	Yes	Yes
10KR	No	No	Yes	Yes
ΔCUE	No	No	No	Yes
mutCUE1,2,3	No	No	No	Yes
ΔCUE-Ub	Yes	Yes	No	Yes/No
mutCUE2-Ub	Yes	Yes	No	Yes/No
10KR-Ub	Yes	Yes	Yes	Yes/No
T68D	Yes	No	Yes	Yes
T68A	Yes	Yes	Yes	Yes
mutLDT1,2	Yes	No	Yes	No

Table 3.

Overview of AUP1 constructs analyzed in this thesis

Different HA-tagged AUP1 constructs analyzed in this thesis with respect to their ubiquitination state, ability to induce LD clustering, integrity of their CUE domain and localization to LDs.

Results shown in this thesis demonstrates that mutation or deletion of the AUP1 CUE domain alone strongly attenuated the ability of AUP1 to promote LD clustering, whereas deletion of the G2BR domain had no effect on the clustering of LDs.

Additionally, it was shown that the AUP1 CUE domain itself is essential for the intramolecular ubiquitination of AUP1. This finding is in line with observations made in HeLa cells, where AUP1 was also found to be ubiquitinated in a CUE dependent

manner (Klemm *et al.*, 2011). UBD dependent ubiquitination has been described for several proteins (Polo *et al.*, 2002; Klapisz *et al.*, 2002; Shiba *et al.*, 2004; Oldham *et al.*, 2002; Hicke *et al.*, 2005b), and has been termed coupled monoubiquitination. Presumably, AUP1 belongs to this larger group of UBD containing proteins, which undergo coupled monoubiquitination. Results presented in this thesis and by others (Klemm *et al.*, 2011) suggest that AUP1 is predominantly modified by one or two ubiquitin moieties, as apparent from a molecular weight shift of AUP1 by 8 and 16kDa, respectively. Yet, it could not be determined whether AUP1 is modified by two ubiquitin moieties at two different lysine residues or by di-ubiquitin attached to one single lysine residue. This question will be addressed in future studies using different His-tagged ubiquitin lysine mutants for the co-purification of AUP1.

How the CUE domain participates in the ubiquitination of AUP1 is as yet not clear. Possibly, a specific E3 ubiquitin ligase interacts with conserved residues within the AUP1 CUE domain and subsequently transfers ubiquitin moieties to AUP1, as described for Eps15 (Fallon *et al.*, 2006b; Woelk *et al.*, 2006) and depicted in Figure 7. Alternatively, an E3 ubiquitin ligase independent mechanism for AUP1 ubiquitination is conceivable. It was shown for several UBD containing proteins that intramolecular ubiquitination can be achieved directly by interaction with an E2 ubiquitin conjugase, and that this process is again dependent on a functional UBD (Hoeller *et al.*, 2007; Sorkin, 2007). Since AUP1 binds the E2 ubiquitin conjugase Ube2g2, it seems feasible that this protein complex alone could catalyze AUP1 ubiquitination. Yet, it was demonstrated in this thesis and elsewhere (Klemm *et al.*, 2011) that an AUP1 truncation mutant lacking the G2BR domain, and hence unable to bind Ube2g2 (Spandl *et al.*, 2011) is still efficiently ubiquitinated. However, a possible trans-ubiquitination of AUP1-ΔG2BR by an endogenous AUP1 Ube2g2 complex cannot be excluded. Continuative research on AUP1 employing *in vitro* ubiquitination assays using bacterial expressed and purified AUP1 and Ube2g2 proteins will contribute to the understanding of the molecular mechanism behind AUP1 ubiquitination.

Furthermore, this thesis demonstrates that AUP1 is ubiquitinated at multiple lysine residues and that AUP1 lacking all lysine residues failes to undergo coupled monoubiquitination. This finding is supported by two recent proteome-wide ubiquitination site surveys, which also found AUP1 to be ubiquitinated at multiple

lysine residues (Wagner *et al.*, 2011; Kim *et al.*, 2011). Contradictory to the finding that the AUP1 CUE domain is important for LD clustering was the observation that LD clustering was strongly attenuated in COS-7 cells overexpressing AUP1 lacking all lysine residues, although it retained the CUE domain. It was therefore speculated whether the ability of AUP1 to promote LD clustering depends on the ubiquitination of AUP1, rather than on a functional CUE domain. This thesis demonstrates that a single ubiquitin moiety fused to AUP1 is sufficient to induce LD clustering, regardless of the presence or absence of the CUE domain. To our best knowledge, ubiquitination is the first posttranslational protein modification described that controls the clustering of LDs. Yet, it must be noted that the AUP1 ubiquitin chimera proteins analyzed in this thesis exhibited a different intracellular distribution than overexpressed full length AUP1. The AUP1 ubiquitin chimera proteins were enriched at the site of LD clusters, yet they exhibited a more diffuse distribution within these LD clusters. For Full length AUP1, characteristic ring-like structures around single LDs within LD clusters were observed. Since AUP1 localizes to both, LDs and the ER, it might be possible that the AUP1 ubiquitin chimera proteins did not actually localize to LD clusters but rather to ER membranes adjacent to these LD clusters. Indeed, a close connection of LDs with ER membranes is a commonly observed structural feature of LDs (Murphy *et al.*, 2009; Beller *et al.*, 2010; Cushman, 1970). However, this thesis demonstrates that AUP1 mutants which are retained within the ER membrane completely lose their ability to promote LD clustering, albeit being still modified by ubiquitin. It can therefore be assumed that the AUP1 ubiquitin chimera proteins have to reach LDs in order to promote their clustering. Yet, further experiments will be necessary to exactly determine the localization of the AUP1 ubiquitin chimera proteins. For future studies it will also be necessary to analyze whether the orientation of ubiquitin fused to AUP1 influences the induced clustering of LDs. In all AUP1 ubiquitin fusion proteins generated for this thesis, ubiquitin is fused with its N-terminus to the C-terminus of AUP1. However, ubiquitin is usually conjugated to a lysine residue within target protein through its C-terminal glycine residue, whereas the N-terminus protrudes from target protein (Johnson, 2002; Welchman *et al.*, 2005; Wickliffe *et al.*, 2011). Ubiquitin is therefore fused to AUP1 in a backward orientation. It should be analyzed whether ubiquitin fused to the N-terminus of AUP1 also promotes the clustering of LDs.

A further question that remains unanswered is whether ubiquitin fused to any LD associated protein would also suffice to induce LD clustering. In an attempt to answer this question, ubiquitin was fused to either the N- or C-terminus of the LD associated protein NSDHL and overexpressed in COS-7 cells. Yet, expression levels of HA-tagged NSDHL ubiquitin chimera proteins were extremely low and localization to LDs almost undetectable and inconsistent (Fig. 27, B), whereas HA-tagged NSDHL alone localized efficiently to LDs (Fig. 27, A). Even though NSDHL ubiquitin chimera proteins were unable to promote LD clustering, their inconsistent localization to LDs made it impossible to draw a conclusion as to whether ubiquitin fused to NSDHL might be capable of promoting LD clustering. Therefore, at this point it cannot be excluded that AUP1 fused to ubiquitin serves as an artificial vehicle for targeting a monoubiquitin to LDs, and that monoubiquitin targeted to LDs is as such sufficient to promote LD clustering. Any assumption as to how the ubiquitination of AUP1 might promote LD clustering must be speculative.

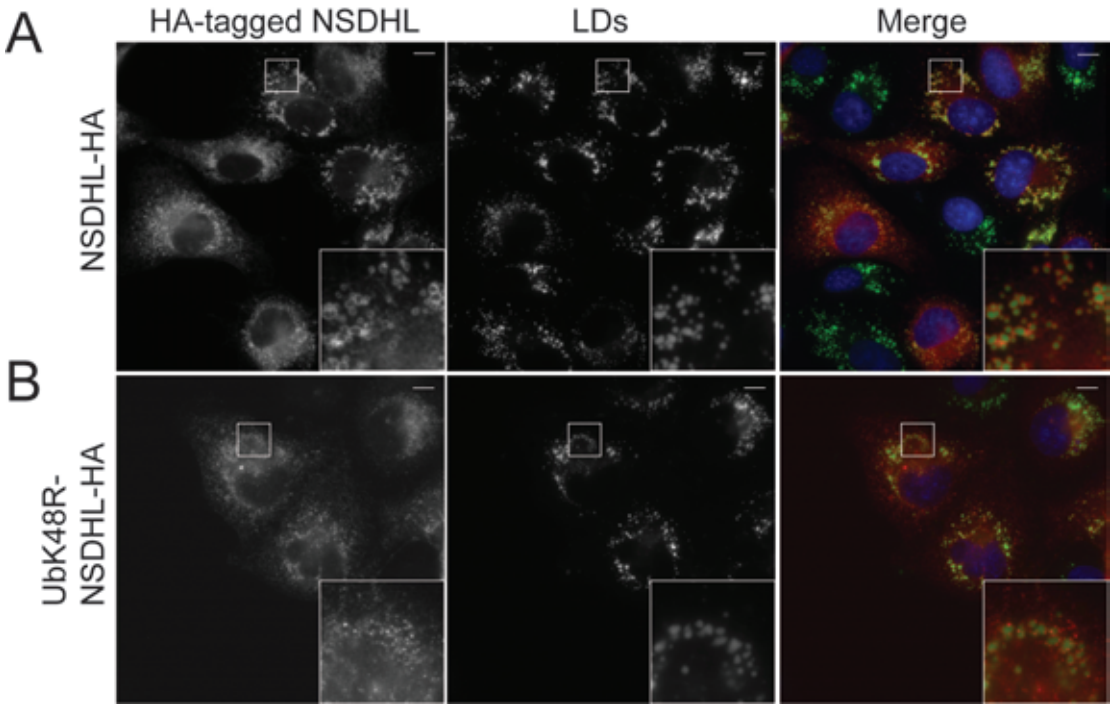

Figure 27.

Ubiquitin fused to the LD associated protein NSDHL

(A-B) Fluorescence micrographs of COS-7 cells overexpressing HA-tagged NSDHL constructs (as indicated) and supplemented with 50 µ M oleate are shown. Cells were immunostained with anti-HA antibody (left panels). LDs were stained with LD540 (middle panels). Merged images with nuclei stained by DAPI in blue, NSDHL in red and LDs in green are shown (right panels). Bars, 10µm. NSDHL-HA efficiently localized to LDs and did not induce LD clustering. Ubiquitin fused to the N-terminus of NSDHL did not show consistent co-localization with LDs.

5.3. Membrane tethering

In principal, the smallest entity of an LD cluster consists of two individual LDs that are tethered by an as yet unknown molecular mechanism. This tethering of individual LDs can be thought of as being reminiscent to the tethering between distinct membranous bound cellular compartments. Up to date numerous proteins, called tethering factors, have been identified, which participate in membrane-membrane tethering events (Lupashin and Sztul, 2005; Sztul and Lupashin, 2009; Yu and Hughson, 2010). These tethering factors are a diverse group of mainly peripherally

associated membrane proteins, which form physical links between two each other opposing membranes (Brocker *et al.*, 2010; Sztul and Lupashin, 2009; Yu and Hughson, 2010). Membrane tethering can be mediated by homo-oligomerization of proteins, as proposed for the Golgi reassembly and stacking proteins (GRASPs) 55 and 65, which are thought to be involved in the stacking of Golgi cisternae by tethering Golgi membranes from opposing cisternae (Wang *et al.*, 2003; Xiang and Wang, 2010; Sengupta *et al.*, 2009; Lowe, 2011; Tang *et al.*, 2010). Membrane tethering can also be achieved by the assembly of large multisubunit complexes, as proposed for the transport protein particle I (TRAPPI) complex. The TRAPPI complex has been suggested to mediate tethering of coat protein complex II (COPII) coated vesicles to the Golgi membrane (Sacher *et al.*, 2001; Cai *et al.*, 2007; Conibear, 2011). The underlying principle governing membrane-membrane tethering is therefore thought to be mediated by the interaction between proteins on themselves opposing membranes. Figure 28 shows a schematic representation of several proposed molecular mechanisms for membrane-membrane tethering events.

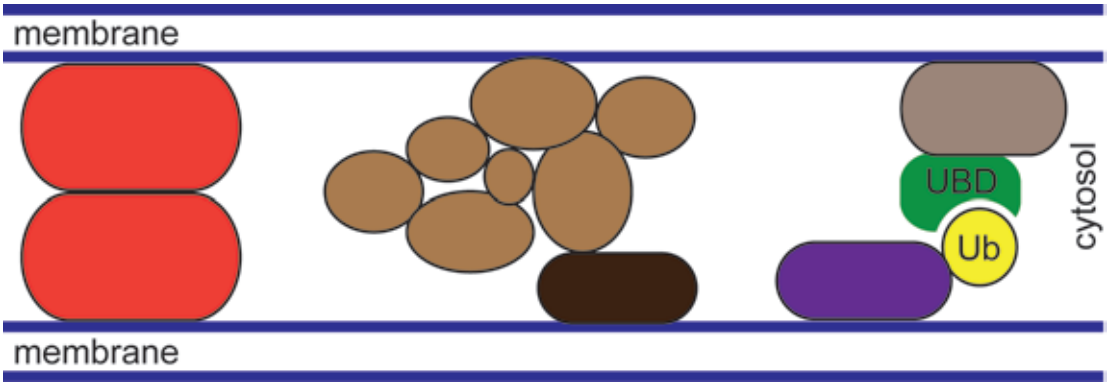

Figure 28.

Membrane-membrane tethering

Schematic representation of different membrane-membrane tethering events in cells are shown. Left: Homodimerization of proteins attached to each other opposing membranes, as suggested for GRASP proteins. Middle: Assembly of a multisubunit protein complex to tether each other opposing membranes, as suggested for the TRAPPI complex. Right: A cytosolic UBD containing protein simultaneously binds a ubiquitinated membrane protein and another membrane bound protein on an opposing membrane, as suggested for SQSTM1/p62.

Indeed, the dimerization of proteins from opposing LDs as a mean to drive LD clustering has been suggested for perilipin A (Brasaemle *et al.*, 2000), caveolin-1 (Ostermeyer *et al.*, 2001) and fsp27 (Jambunathan *et al.*, 2011). fsp27 has been shown to homodimerize and heterodimerize with CIDEA (cell death-inducing DFFA-like effector a) (Liu *et al.*, 2009). The C-terminus of CIDEA itself has also been shown to promote the clustering of LDs (Christianson *et al.*, 2010), suggesting that heterodimerization of these two proteins might promote LD clustering. Yet, experimental evidence to prove that protein dimerization might be responsible for LD clustering is lacking. Nevertheless, it can be envisioned that a similar mechanism is applicable to AUP1-induced LD clustering, in which AUP1 on one LD binds directly or indirectly a protein on an opposing LD and thereby physically tethers these LDs. The ubiquitination of AUP1 would somehow participate and potentiate this tethering event.

5.4. Ubiquitin and membrane tethering

Ubiquitination of membrane proteins has been suggested to be important for the tethering of mitochondria to forming autophagosomes in the early steps of mitophagy (Komatsu and Ichimura, 2010; Wild and Dikic, 2010). It has been demonstrated that the cytosolic ubiquitin binding protein of 62kDa/sequestosome1 (P62/SQSTM1) is enriched on ubiquitinated mitochondria (Lee *et al.*, 2010; Ding *et al.*, 2010; Geisler *et al.*, 2010; Huang *et al.*, 2011). P62/SQSTM1 itself contains a UBD, which has been shown to bind ubiquitin (Vadlamudi *et al.*, 1996; Long *et al.*, 2008) and an LC3 interacting region (LIR) which has been demonstrated to bind autophagosomal localized LC3 (microtubule-associated protein-1 light chain 3) (Pankiv *et al.*, 2007; Johansen and Lamark, 2011). P62/SQSTM1 has therefore been suggested to function as a tethering factor that physically links mitochondria to forming autophagosomes in the presence of ubiquitinated mitochondrial proteins (Kirkin *et al.*, 2009; Wild and Dikic, 2010; Komatsu and Ichimura, 2010).

Noteworthy, recruitment of p62/SQSTM1 to ubiquitinated mitochondria has been

observed to promote the clustering of these mitochondria (Okatsu *et al.*, 2010; Narendra *et al.*, 2010). Furthermore, it has been shown that p62/SQSTM1 self-oligomerizes (Lamark *et al.*, 2003) and that mutation of amino acid residues of p62/SQSTM1 important for oligomerization allenuates the clustering of mitochondria (Okatsu *et al.*, 2010). Hence, it has been proposed that oligomerized p62/SQSTM1 can simultaneously bind several ubiquitinated mitochondrial proteins via their UBDs and thereby tether individual mitochondria (Okatsu *et al.*, 2010). Whether p62/SQSTM1 or another protein performs a similar function in the tethering of individual LDs will have to be determined by future studies. Such molecular players might be revealed by pull-down approaches using AUP1 ubiquitin chimera proteins as bait.

Interestingly, it was found that in cells overexpressing spartin, orthologues of p62/SQSTM1 from R*attus norvegicus*, namely zinc transporter (ZIP) 1, 2 and 3 are recruited to LDs (Urbanczyk and Enz, 2011). Although the effect of spartin overexpression on LD clustering was not explicitly studied, fluorescence microscopy pictures from cells overexpressing spartin clearly showed clustering of LDs when adorned by spartin (Urbanczyk and Enz, 2011).

In conclusion, interaction between ubiquitinated proteins and UBD containing proteins could represent a possible mechanism by which membranes are tethered. Based on this assumption, possible models are presented in the next chapter as to how ubiquitinated AUP1 might promote LD clustering.

5.5. Molecular mechanisms for AUP1-induced LD clustering

A very simple model that could explain how monoubiquitinated AUP1 controls LD clustering is depicted in Figure 29. In this model, two monoubiquitinated AUP1 proteins face each other on opposing LDs, and both AUP1 proteins bind the ubiquitin attached to the other protein *in trans* via their CUE domain (henceforward named 'AUP1 homodimerization model'). Although the interaction between ubiquitin and UBDs is thought to be rather weak, with a K_d of around 100 µM (Hicke *et al.*, 2005a;

Hurley *et al.*, 2006), the simultaneous interaction of several AUP1 proteins on opposing LDs might confer a net interaction that is strong enough to tether LDs.

Alternatively, ubiquitin attached to AUP1 could bind a UBD from another so far uncharacterized protein on an opposing LD (Fig. 29). In this context, Fas associated factor family member 2 (FAS2/UBXD8) contains an UBD (termed ubiquitin-associated (UBA) domain) and has been identified on LDs (Zehmer *et al.*, 2009a). FAS2/UBXD8 has been found to co-precipitate with AUP1 in a pull-down experiment (Klemm *et al.*, 2011), and was found together with AUP1 in a purified LD fraction from A431 cells (Moessinger *et al.*, 2011). Furthermore, it was shown that overexpression of UBX2, a close homologue of FAF2/UDXB8 in yeast, caused aggregation of LDs (Wang and Lee, 2012). However, deletion of the UBA domain of UBX2 did not disrupt the aggregation of LDs (Wang and Lee, 2012). In addition to the UBA domain, FAS2/UBXD8 also contains a ubiquitin regulatory X (UBX) domain. UBX domains have been shown to share the same superfold as ubiquitin (Buchberger *et al.*, 2001; Yuan *et al.*, 2001). It has also been demonstrated that protein domains, which exhibit the same superfold as ubiquitin, can interact with UBDs (Mueller and Feigon, 2003; Zhang *et al.*, 2009). In the context of LD tethering, it can be envisioned that the UBX domain of FAS2/UBXD8 interacts with the CUE domain of AUP1; thereby tethering LDs. Interaction studies of different AUP1 mutants, lacking ubiquitination or the CUE domain, with FAS2/UBXD8 might reveal a functional connection between AUP1 and FAS2/UBXD8.

Analog to p62/SQSTM1 mediated tethering of mitochondria to autophagosomes (Kirkin *et al.*, 2009). AUP1 ubiquitination might recruit a cytosolic UBD containing protein, which simultaneously binds to another LD associated protein and hence could function as a physical link between individual LDs (Fig. 29). Such a UBD containing protein could also exist in an oligomerized state, as described for p62/SQSTM1, and as such bind to several ubiquitinated AUP1 proteins on different LDs, thereby tethering these LDs.

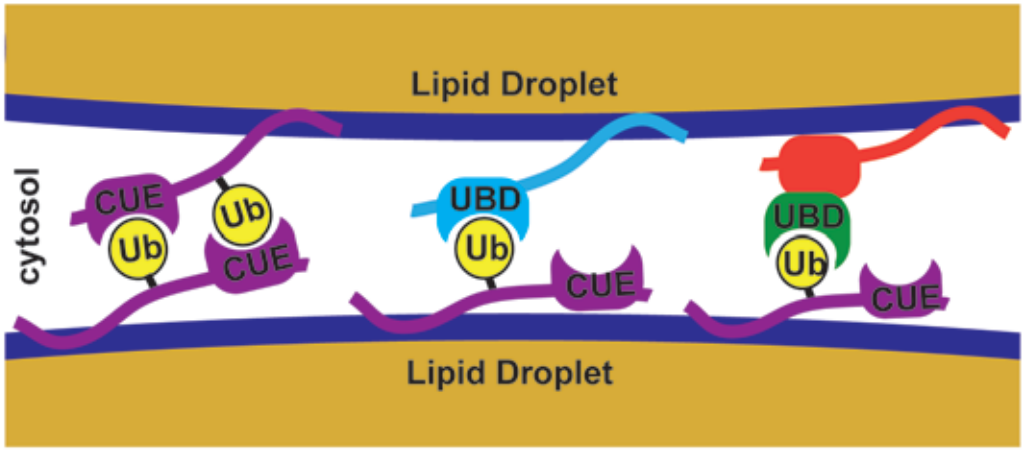

Figure 29.

Possible molecular mechanisms explaining AUP1-dependent LD clustering

Schematic representation depicting possible molecular mechanisms for AUP1-induced LD clustering. Left: Two AUP1 proteins face each other on opposing LDs. Both AUP1 proteins bind the ubiquitin attached to the other protein *in trans* via their CUE domain. Middle: Ubiquitin attached to AUP1 is bound by a UBD containing protein from an opposing LD. Reight: A cytosolic UBD containing protein binds ubiquitin attached to AUP1 and simultaneously to a protein from an opposing LD.

Several lines of evidence presented in this thesis support the above models. Mainly that AUP1 is ubiquitinated and that monoubiquitination of AUP1 is sufficient to promote LD clustering. It was further demonstrated in this thesis that AUP1 can interact with itself. On the other hand, several experiments conducted in this thesis are contradictory to these models.

First, overexpression of AUP1 with a deleted CUE domain fused to ubiquitin (AUP1-ΔCUE-Ub) caused the clustering of LDs. In this case, LD clustering cannot be explained by the AUP1 homodimerization model. AUP1 ubiquitin chimera proteins lacking their CUE domains would be unable to interact with ubiquitin. Yet, COS-7 cells also express endogenous AUP1, which could bind ubiquitin fused to AUP1-ΔCUE and thereby provide enough dimerization events to tether LDs. Repeating the same experiment in an AUP1 knock-out background would help to clarify this issue

(see 5.10.).

Second, it was shown that interaction of AUP1 with itself is not abolished by deletion of the CUE domain. This result indicates that AUP1 self-interaction does not rely on a functional CUE domain or the ubiquitination of AUP1. The pull-down assays to generate these data should be repeated, employing different combinations of myc- and HA-tagged AUP1 mutants. Likely, additional mutational analysis of AUP1 will be necessary to reveal regions of AUP1 that are important for AUP1 self-interaction. Such mutational studies will help to clarify the mechanism for AUP1 self-interaction, and a possible connection to the clustering of LDs.

Third, ubiquitin UBD interactions rely on conserved amino acid residues on ubiquitin, mainly isoleucine 44 and aspartic acid 58. It was shown in this thesis that mutation of either of these two residues to alanine on ubiquitin fused to AUP1-ΔCUE did not obviate LD clustering. These results suggest that interaction between ubiquitin and UBDs, which rely on these conserved amino acid residues are not required for AUP1-induced LD clustering. However, exchanging the hydrophobic amino acid isoleucine 44 by the hydrophobic amino acid alanine might not suffice to efficiently disrupt binding of ubiquitin to a UBD. Yet, the same mutation on ubiquitin has been demonstrated by several studies to disrupt binding of ubiquitin to a diverse range of UBDs (Shih *et al.*, 2003; Shih *et al.*, 2002; Kang *et al.*, 2003). In addition, other amino acid residues on ubiquitin might be important for binding to UBDs. Indeed, a ubiquitin-binding motif (UBM) from polymerase iota (poli) interacts with amino acid residues on ubiquitin centered around leucine 8 (Bienko *et al.*, 2005). The zinc-finger ubiquitin binding domain (ZnF UBP) of deubiquitinating enzyme isopeptidase T (isoT) contacts hydrophobic amino acid residues leucine 8 and isoleucine 36 on ubiquitin (Reyes-Turcu *et al.*, 2006). Further mutational analysis of ubiquitin fused to AUP1-ΔCUE will be necessary to determine amino acid residues on ubiquitin that are important for promoting LD clustering.

In conclusion, several mechanisms explaining how the ubiquitination of AUP1 could promote LD clustering have been discussed. Yet, further research will be necessary to determine whether any of the presented models contribute to this process.

5.6. A function to LD clustering

As the molecular mechanism for LD clustering is still elusive, so is the function of LD clustering. So far only few suggestions have been made as to why LD clusters are formed.

It was suggested that fsp27-induced LD clustering is a prerequisite for LD fusion (Jambunathan *et al.*, 2011). fsp27 has been shown to concentrate at discrete contact sites between individual LDs and it was observed that fluorescent labeled fatty acids transit from one LD to another, resulting in a mixed population of enlarged and small LDs. This phenomenon was only observed when fsp27 was correctly targeted to LDs (Gong *et al.*, 2011). How precisely lipids are transferred from one LD to another is as yet unknown. Suggestions range from pores connecting individual LDs to proteins shuttling lipids between LDs (Gong *et al.*, 2011) and actual fusion of LD membranes mediated by SNARE proteins (Bostrom *et al.*, 2007). That LDs clustered by AUP1 undergo actual fusion is rather unlikely as single LDs could be observed within these LD clusters. A possible molecular machinery necessary for LD fusion should be present in COS-7 cells, as fsp27 mediated LD fusion was studied in COS-7 cells (Jambunathan *et al.*, 2011). The selective transfer of lipids from one LD to another is also unlikely, as LDs within these clusters seemed rather homogenous in size.

perilipin A is another LD associated protein which has been studied in more detail with respect to its ability to promote LD clustering. Ectopic expression of perilipin A in fibroblastic 3T3-L1 pre-adipocytes has been observed to cause LD clustering (Brasaemle *et al.*, 2000; Marcinkiewicz *et al.*, 2006). Furthermore, ectopic expression of perilipin A in fibroblastic 3T3-L1 pre-adipocytes also caused a strong increase in cellular TAG storage (Brasaemle *et al.*, 2000). It is assumed that this increase in TAG storage is mediated by an intrinsic function of perilipin A to shield LDs from cytosolic lipases (Brasaemle *et al.*, 2000; Marcinkiewicz *et al.*, 2006). However, it has also been speculated whether the clustering of LDs as such is a mechanism to prevent lipolysis (Christianson *et al.*, 2010; Garcia *et al.*, 2004; Marcinkiewicz *et al.*, 2006). Whether AUP1-induced LD clustering increases TAG content in cells is currently under investigation. Initial experiments using clickable fatty acids suggested that the ability of AUP1 to promote LD clustering did not positively correlate with an

increased TAG content in cells (Lohmann, Daniel and Piotrowitz, Kira). However, a study from Klemm et al. suggests a slight increase in neutral lipid content in HeLa cells overexpressing AUP1. Yet, it was suggested that the AT domain of AUP1 is important for this increase in neutral lipid storage, whereas the CUE domain seemed dispensable (Klemm *et al.*, 2011).

5.7. Phosphorylation and LD declustering

Another finding from studies on perilipin A was that the clustering of LDs in fibroblastic 3T3-L1 pre-adipocytes ectopically expressing perilipin A is reversible upon lipolytic stimulation (Marcinkiewicz *et al.*, 2006). perilipin A itself is phosphorylated during this event and it has been shown that the mutation of one proposed phosphorylation site within perilipin A is sufficient to prevent the declustering of LDs upon lipolytic stimulation (Marcinkiewicz *et al.*, 2006). In adipocytes, Perilipin A is phosphorylated upon β -adrenergic receptor pathway induced lipolysis (Brasaemle, 2007) and lipolytic stimulation of adipocytes leads to the fragmentation of few large LDs into numerous dispersed and declustered LDs (Londos *et al.*, 1999; Marcinkiewicz *et al.*, 2006). Posttranslational modification of perilipin A by phosphorylation has been therefore suggested to actively disperse clustered LDs (Marcinkiewicz *et al.*, 2006).

Interestingly, it was found that phosphorylation of a *Drosophila* homologue of perilipin A, namely LSD2 influences the intracellular motility of LDs (Welte *et al.*, 2005). It has been suggested that microtubule and motor protein dependent motility of LDs might be a necessary force to pull LDs apart and could hence be a mean to actively disperse LD clusters (Marcinkiewicz *et al.*, 2006; Welte *et al.*, 2005; Welte, 2009). Based on these observations it was proposed that phosphorylation of perilipin A might enable active transport of LDs and hence cause the dispersion of LD clusters (Marcinkiewicz *et al.*, 2006).

Strikingly, this thesis demonstrated that an AUP1 Thr68 phosphomimetic mutant quantitatively reduced the ability of AUP1 to induce LD clustering. This result suggests that AUP1 phosphorylation at Thr68 might be a posttranslational

modification that negatively influences AUP1-dependent LD clustering. However, at this point it cannot be distinguished whether the AUP1 Thr68 phosphomimetic mutant prevents the clustering of LDs or actively promotes the declustering of previously clustered LDs. Results from this thesis only indicate that phosphorylation of AUP1 at Thr68 does not prevent LD clustering by hindering AUP1 ubiquitination.

Taken together, results from this thesis suggest that the posttranslational modification of AUP1 might regulate LD clustering in two ways. First, the ubiquitination of AUP1 promotes the clustering of LDs and second, the subsequent phosphorylation of AUP1 Thr68 promotes the dispersion of these clusters. Also, posttranslational modifications of proteins by ubiquitination or phosphorylation are readily reversible (Amerik and Hochstrasser, 2004; Deribe *et al.*, 2010). Cellular deubiquitinating enzymes or phosphatases might counteract AUP1 ubiquitination and phosphorylation, respectively. A model depicting how the posttranslational modifications of AUP1 might control intracellular LD clustering, based on the above assumptions, is shown in Figure 30.

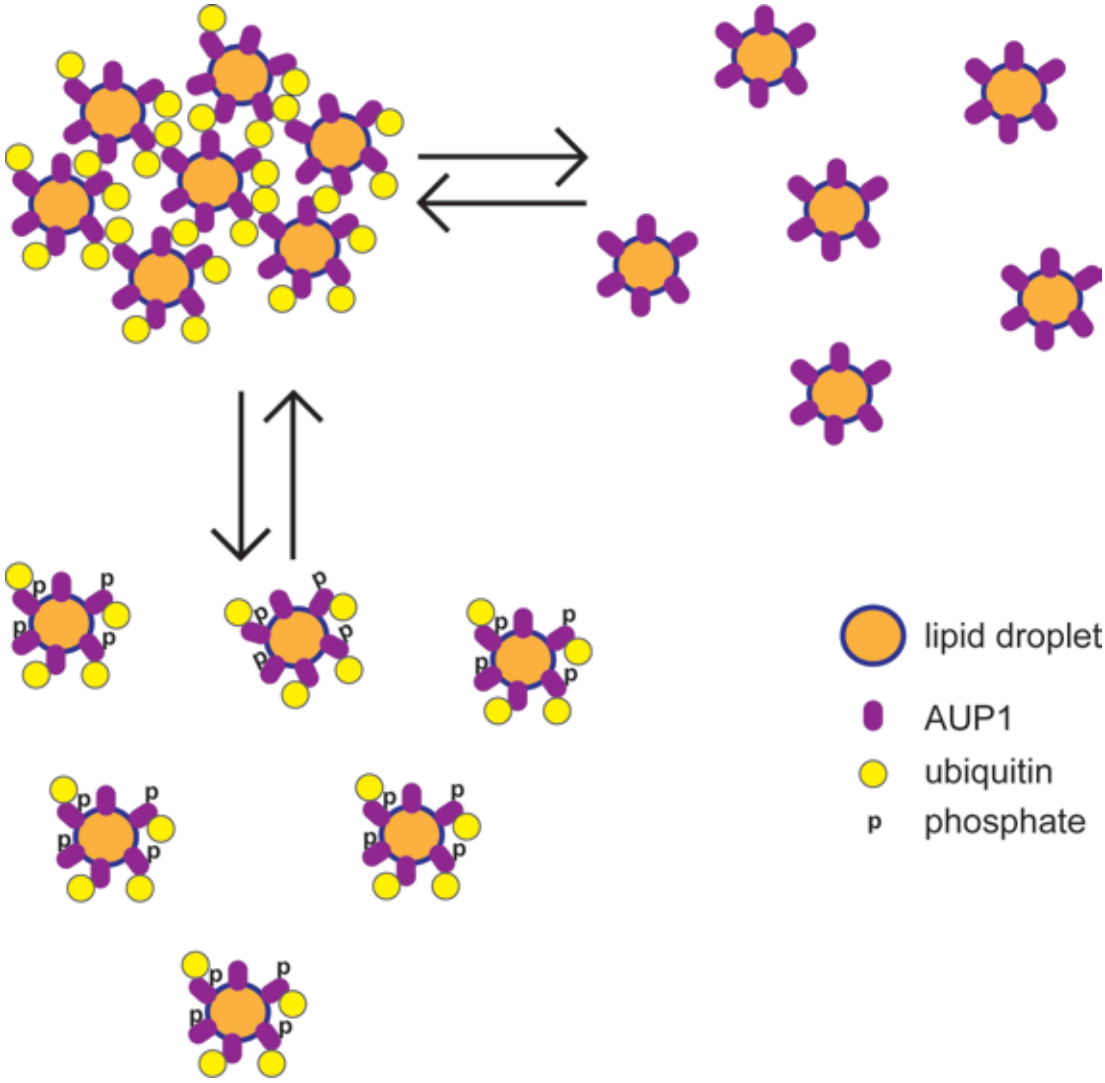

Figure 30.

LD clustering under the control of AUP1

Ubiquitination of AUP1 promotes the clustering of LDs whereas phosphorylation of ubiquitinated AUP1 actively promotes declustering of LDs. Both processes might also be reversible by the activity of deubiquitinating enzymes and phosphatases, respectively.

It is tempting to speculate whether the posttranslational modifications of AUP1, and hence the intracellular distribution of LDs, are governed by upstream signaling events, as has been suggested for the phosphorylation of perilipin A. Future studies will show whether AUP1-induced LD clustering might be reversible under certain signaling conditions.

5.8. AUP1 ubiquitination in a different context

So far it was only speculated as to how AUP1 ubiquitination influences the clustering of LDs. However, ubiquitination of AUP1 might also control other aspects of the proposed molecular functions of AUP1. It was shown that AUP1 has weak acyltransferase activity, which might be impaired or enhanced by ubiquitination of AUP1. Negative regulation of enzymatic activity through conformational changes induced by ubiquitination has for example been described for the deiodinase, iodothyronine, type II (DIO2) enzyme (Sagar *et al.*, 2007).

Furthermore, AUP1 has been suggested to function in ERAD by binding to ER quality control machinery and dislocation substrates (Spandl *et al.*, 2011; Klemm *et al.*, 2011; Mueller *et al.*, 2008; Claessen *et al.*, 2010), and it has been shown that mutation of the CUE domain diminishes binding to these components (Klemm *et al.*, 2011). How and whether the ubiquitination of AUP1 itself influences the role of AUP1 in ERAD is as yet an open question.

As outlined during this discussion, ubiquitination of mitochondrial membrane proteins has been suggested to be important for their autophagocytosis (Kuma and Mizushima, 2010). Ubiquitination of AUP1 might therefore be a possible signal for autophagosomal clearance of LDs (Singh and Cuervo, 2012). Indeed, recent studies demonstrated that LDs are found within autophagic vacuoles and that the autophagosomal marker protein LC3 accumulates at LDs (Rabinowitz and White, 2010; Singh *et al.*, 2009; Kaini *et al.*, 2012). Following autophagocytosis in cells overexpressing the AUP1 ubiquitin chimera proteins might reveal the importance of ubiquitinated LD proteins for their autophagosomal degradation.

5.9. LD clusters, a shelter for proteins

Finally, a speculative thought to the function of AUP1-induced LD clustering based on different themes discussed in this thesis.

Under sufficient nutritional conditions, AUP1 might be ubiquitinated and subsequently recruit UBD containing proteins, like p62/SQSTM1 to LD clusters. These LD clusters might serve as a site for the sequestration of proteins that normally function in different cellular processes, thereby keeping them away from their intracellular location of action; and hence in an inactive state. Under nutritional deprivation, AUP1 might undergo regulated phosphorylation, promoting the declustering of these LD clusters and the release of sequestered proteins. Released proteins would then be quickly available to respond to the change in nutritionally conditions. P62/SQSTM1 could for example engage in autophagocytosis in order to supply cells with necessary energy. At least for fsp27 it was shown that partitioning of fsp27 onto LDs attenuates its function in promoting cell death (Liu *et al.*, 2009). Yet, whether the ability of fsp27 to induce LD clustering coincides with the attenuated apoptotic function of fsp27 was not analyzed.

However, LDs have been found to function as sites for the sequestration of several proteins normally not found to be associated with LDs (Leber *et al.*, 1998; Jiang *et al.*, 2007; Whitehead *et al.*, 2004; Li *et al.*, 2012; Cermelli *et al.*, 2006), and it has been suggested that LDs might more generally serve as a temporary shelter for specific proteins (Welte, 2007). Whether the clustering of LDs participates in the sequestration of such protein awaits further studies.

5.10. AUP1 specific Zinc Finger Nucleases

As mentioned at several points during this discussion, endogenous background levels of AUP1 expression hampered the interpretation of some results presented here. As part of this thesis and in collaboration with a diploma (Myriam Sarmiento Diaz) and master student (Wunderling Klaus), standard protocols for the generation of AUP1

knock-out cell lines using zinc finger nuclease (ZFN) technology were used in a first trial to analyze efficiency of AUP1 specific ZFNs.

ZFNs are hybrid enzymes of the FokI-cleavage domain and several DNA-binding zinc finger motives, which are able to introduce a broad range of genome alterations in eukaryotic cells (Bibikova *et al.*, 2003; Porteus and Carroll, 2005; Wu *et al.*, 2007). We obtained two ZFNs (Sigma-aldrich) which specifically bind to genomic AUP1. These AUP1 specific ZFNs were used in a first trial to generate an AUP1 knock-out in A431 cells (results will be published elsewhere). The AUP1 ZFNs are currently used to generate an AUP1 knock-out cell line.

6. Conclusion and Outlook

In conclusion, results from this thesis confirmed and extended initial observations that AUP1 is a novel protein involved in the intracellular clustering of LDs. It was shown that AUP1 undergoes intramolecular ubiquitination in a CUE dependent manner and that this ubiquitination of AUP1 is a prerequisite to induce the clustering of LDs. Furthermore, results from this thesis suggest that AUP1 phosphorylation at Thr68 promotes the dispersion of LD clusters. Posttranslational modifications of AUP1 through ubiquitination and phosphorylation could therefore represent a possible molecular mechanism to control the intracellular distribution of LDs.

During the work on this thesis a large set of diverse AUP1 mutants were generated which will help to tackle immediate questions of how AUP1 controls the intracellular distribution of LDs. How does ubiquitin attached to AUP1 promote the clustering of LDs? Which role does AUP1 play in this context? Does the observed dimerization of AUP1 function in the clustering of LDs? Further mutational analysis of ubiquitin fused to AUP1 will provide additional cues as to the function of ubiquitin in LD clustering. Additional molecular players involved in the clustering of LDs might be revealed by pull-down approaches using AUP1 ubiquitin chimera proteins as bait. Also, the molecular mechanism of AUP1 ubiquitination will have to be determined by in vitro ubiquitination assays. It will be of general interest to determine whether the posttranslational modifications of AUP1 and hence the intracellular distribution of LDs are governed by upstream signaling events. In this context, finding the kinase which phosphorylates AUP1 at Thr68 might provide important cues for further research on possible upstream signaling events controlling the posttranslational modifications of AUP1.

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8. Publication Record

Data and work from thesis have been published or will be published in the following articles:

- Johanna Spandl, Daniel Lohmann, Lars Kuerschner, Christine Moessinger and Christoph Thiele (2011). "Ancient Ubiquitous Protein 1 (AUP1) Localizes to Lipid Droplets and Binds the E2 Ubiquitin Conjugase G2 (Ube2g2) via Its G2 Binding Region". The Journal of Biological Chemistry
- Thiele C, Papan C, Hoelper D, Kusserow K, Gaebler A, Schoene M, Piotrowitz K, Lohmann D, Spandl J, Stevanovic A, Shevchenko A, Kuerschner L. (2012). "Tracing Fatty Acid Metabolism by Click Chemistry". ACS Chemical Biology
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"That is one small step for mankind…one… giant leap for me" – Freely modified from Neil Armstrong