TOR complex 2 regulates plasma membrane homeostasis

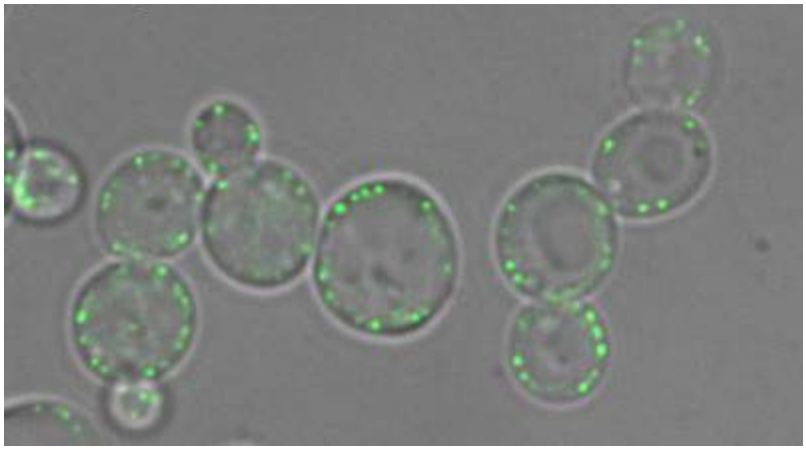

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TABLE OF CONTENTS

Summary

The plasma membrane is the boundary of cells. It delimits the cellular interior from its environment, mediates substance transport and the perception of extracellular stimuli. Complex sphingolipids are essential structural components of the plasma membrane and play important roles in cellular signaling. While the synthesis and degradation pathways of sphingolipids are well known, the mechanism how cells perceive and adjust sphingolipid levels in the plasma membrane is poorly understood. The target of rapamycin (TOR) kinase, assembled into TOR complex 2 (TORC2), promotes sphingolipid synthesis by activating Ypk kinases. In addition, TORC2 phosphorylates the plasma membrane-localized Slm proteins, which also positively regulate sphingolipid metabolism. However, the upstream regulation of TORC2 and the molecular function of Ypk kinases and Slm proteins were not known. Therefore, the aims of my thesis were a) to elucidate whether and how TORC2 responds to altered sphingolipid levels, and b) to characterize the role of Slm proteins in relation to Ypk kinase function in the regulation of sphingolipid levels.

Live cell imaging of TORC2 components tagged with GFP revealed that TORC2 localizes to a previously uncharacterized plasma membrane domain, which we termed the membrane compartment occupied by TORC2 (MCT). We discovered that TORC2 plasma membrane localization is essential. We observed that Slm proteins partially colocalize with TORC2 in the MCT, and additionally localize to a distinct plasma membrane compartment termed eisosomes. Localization of Slm proteins to either compartment is dynamic and dependent on sphingolipid levels. Pharmacological inhibition of sphingolipid synthesis triggers the release of Slm proteins from eisosomes. This relocalization is essential and correlates with a dramatic increase in TORC2 activity towards Ypk1. Slm proteins are required for Ypk1 activation and act upstream of TORC2 by facilitating the transient formation of TORC2-Slm-Ypk1 signaling complexes. Activated Ypk1 phosphorylates Orm proteins, leading to an increase in sphingolipid synthesis, which counteracts the lack of complex sphingolipids in the plasma membrane. Additionally, plasma membrane stretch activates Slm-mediated TORC2-Ypk1 signaling. We therefore conclude that TORC2 is part of a feedback mechanism to regulate the availability of sphingolipids and membrane according to cellular needs. The results of my thesis not only shed light on the still poorly characterized upstream regulation of TORC2, but also help to understand how cells maintain plasma membrane homeostasis during osmotic changes and cell expansion.

Introduction

The organization of plasma membrane lipids and proteins

The composition and structure of the plasma membrane

The plasma membrane encloses cells and protects them from the outside environment. It acts as diffusion barrier for water-soluble molecules and is responsible for the perception of extracellular stimuli, such as chemical messengers, and their subsequent conversion into cellular signals. The plasma membrane is able to adapt to changes in environmental conditions, to coordinate secretion and nutrient import, and to keep its homeostasis throughout cell proliferation and division. The complexity of its composition facilitates the regulation of such diverse tasks: The plasma membrane is composed of thousands of different lipid species (Guan and Wenk, 2006; Shevchenko and Simons, 2010) and hundreds of different proteins interacting with these lipids (Figure 1).

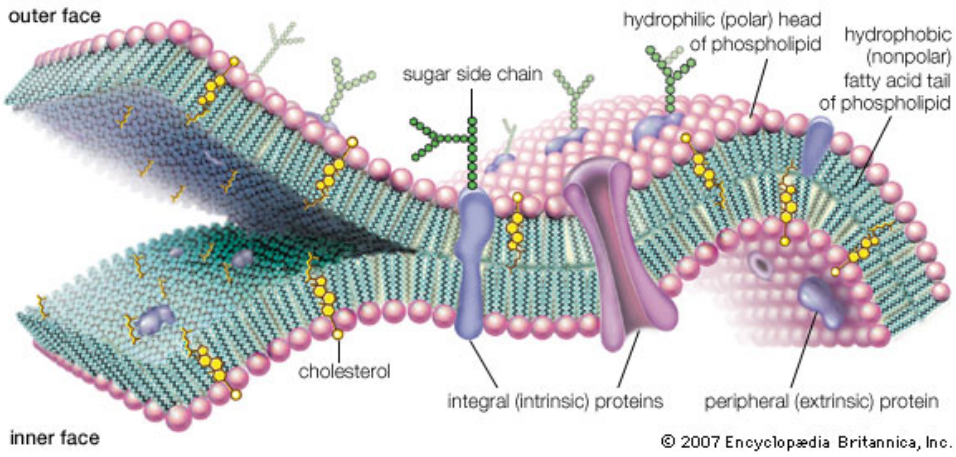

Figure 1: The structure of the plasma membrane. The plasma membrane is a bilayer composed of an intricate mixture of phospholipids and diverse integral and peripheral proteins. (Picture taken from *Encyclopædia Britannica Online*.)

The lipid composition of the plasma membrane

Lipids provide the structural basis of all biological membranes and can be assigned to three major classes: glycerophospholipids, sphingolipids, and sterols (Figure 2). 70 to 80 percent of all phospholipids belong to the class of glycerophospholipids (van Meer *et al.*, 2008). Glycerophospholipids possess a hydrophobic part composed of saturated and/or unsaturated

fatty acid chains attached to glycerol, and a variable hydrophilic head group. The largest subgroups of glycerophospholipids are represented by phosphatidylcholine, which accounts for about 40 percent of all phospholipids in mammalian plasma membranes, phosphatidyl-ethanolamine (approx. 20 percent), phosphatidylserine (approx. 10 percent), and phosphatidyl-inositol (PtdIns) (approx. 2-5 percent) (van Meer *et al.*, 2008).

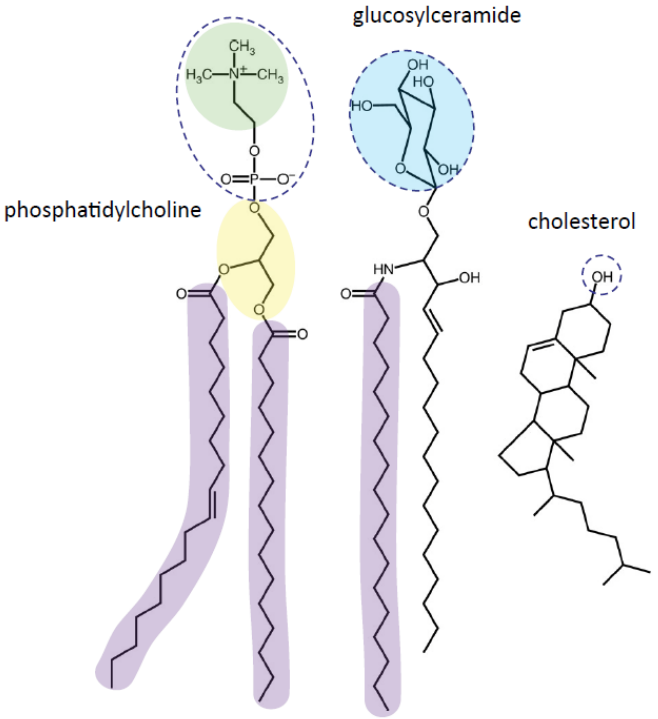

Figure 2: The three main lipid classes in eukaryotic membranes. The chemical structures of glycerophospholipids (phosphatidylcholine, left), sphingolipids (glucosylceramide, middle), and sterols (cholesterol, right) are represented. Hydrophilic parts are encircled (dark blue dashed line). Glycerophospholipids consist of two fatty acids (purple shading) attached to a glycerol (yellow shading). The hydrophilic part of glycerophospholipids consists of a phosphate and different head groups, for instance choline (green shading). Sphingolipids consist of a sphingoid base, which is amide-linked to a saturated fatty acid (purple shading) and a hydrophilic head group, for instance glucose (light blue shading) to form glucosylceramide. Picture modified from (Mansy, 2010).

Sphingolipids belong to the second major lipid class and are structurally different. Their hydrophobic backbone is ceramide, a sphingoid base linked via its amine group to a – typically very long – saturated fatty acid chain. Sphingolipids account for about 25 percent of all plasma membrane lipids. This group can be further divided into phosphosphingolipids, such as sphingomyelin, and glycosphingolipids. The latter are ceramides with carbohydrates as polar head groups (Futerman and Hannun, 2004).

Sterols belong to the third structurally different class of lipids. They are – except for a small hydroxyl group – unpolar and quite flat molecules, which preferably integrate between the hydrophobic fatty acid chains of sphingolipids. Cholesterol is the main sterol to be found in mammalian cells and only represents about one mol percent of all plasma membrane lipids.

Complex sphingolipids are specifically enriched in the plasma membrane as compared to other cellular membranes. Sphingolipids exclusively contain saturated fatty acid chains that can be packed more dense than unsaturated fatty acid chains. The intercalating sterols additionally account for increased membrane packing. Together, the compact packing of both lipids reduces the fluidity and permeability of the plasma membrane and increases its rigidity. This physical property allows the plasma membrane to tolerate mechanical stress, to which it is frequently exposed, for instance during cell growth.

The lipid composition of the plasma membrane differs between cell types and species. The yeast *Saccharomyces cerevisiae*, for example, features ergosterol instead of cholesterol. Though ergosterol appears to be the most abundant lipid species in yeast (12 mol percent of the total lipidome, (Ejsing *et al.*, 2009)) it only accounts for 0.5 mol percent of all plasma membrane lipids (van Meer *et al.*, 2008). The yeast plasma membrane contains much less phosphatidylcholine (approx. 10 percent), but instead comprises elevated levels of PtdIns (approx. 20 percent), phosphatidylserine (also approx. 20 percent) and sphingolipids (approx. 35 percent) (van Meer *et al.*, 2008). Furthermore, the head groups of yeast complex sphingolipids contain inositol phosphate hence differ from mammalian complex sphingolipids containing choline phosphate.

The asymmetrical structure of the plasma membrane

Due to their amphiphatic properties, phospholipids spontaneously self-assemble in aqueous solutions into a 6-10 nanometer thin lipid bilayer. The polar head groups of the phospholipids point outward and their hydrophobic tails point toward the center of the bilayer. The lipid bilayer is asymmetrical in terms of its lipid composition. Complex sphingolipids and phosphatidylcholine predominantly locate to the outer membrane leaflet while phosphatidylethanolamine, phosphatidylserine and PtdIns are enriched in the cytosolic leaflet (van Meer *et al.*, 2008). Most lipids are able to spontaneously “flip” from one leaflet into the

other, but this movement is very slow (Kornberg and McConnell, 1971). Specific enzymes called flippases catalyze the unidirectional flipping of lipid molecules from one leaflet to the other, thus actively maintain the asymmetry of the bilayer.

The recruitment of peripheral proteins to the plasma membrane also differs between the inner and outer leaflet. Glycosylphosphatidylinositol (GPI), for instance, is posttranslationally attached to the carboxyl-terminus of nascent proteins in the ER lumen, and anchors the protein exclusively to the outer leaflet of the plasma membrane. In contrast, other lipid anchors enable the association of proteins with the inner leaflet of the plasma membrane. Prenylation, for instance, is the enzymatic attachment of prenyl groups to a cysteine within a certain amino acid sequence motif (termed CAAX box) at the protein's carboxyl-terminus, and targets the protein to the cytosolic leaflet.

At the inner plasma membrane leaflet, PtdIns can be reversibly mono-, di- and triphosphorylated at the hydroxyl groups of their inositol ring. The resulting phosphoinositides, such as PtdIns(4,5)P_2, represent only about one percent of the plasma membrane lipids (Ferrell and Huestis, 1984) but play an essential role in the regulation of cell signaling as they recruit a plethora of protein effectors to the cytosolic leaflet of the plasma membrane (Kutateladze, 2010). The most prominent protein domain binding to phosphoinositides is the pleckstrin homology (PH-) domain (Wang *et al.*, 1995). The amino acid sequence of this domain varies considerably but its structure is conserved and identified in hundreds of proteins so far. However, most PH-domains exhibit low binding affinity and little specificity to phosphoinositides arguing that PH-domains contribute to the plasma membrane targeting, but may not be the only mechanism for specific recruitment (Yu *et al.*, 2004).

The lateral organization of the plasma membrane

A number of different models were proposed to describe the appearance of the dynamic mixture of lipids and proteins in the plasma membrane. In 1972, Singer and Nicolson suggested that all membrane phospholipids diffuse freely and form two-dimensional fluids, into which transmembrane proteins are integrated (Singer and Nicolson, 1972). According to this "fluid mosaic" model, proteins are supposed to float like boats on the sea of phospholipids only interacting with the lipids directly surrounding them. In contrast, our current understanding of

the lateral organization of the plasma membrane also considers the tendency of specific lipids to cluster and form so-called lipid rafts (Lingwood and Simons, 2010), the local restriction of lipids and proteins by cortical cytoskeleton fences (Kusumi *et al.*, 1993), and the formation of stable domains by protein-protein and protein-lipid interactions (Douglass and Vale, 2005).

Lipid rafts and caveolae represent microdomains

Based on their phase behavior, some sphingolipids and interjacent cholesterols cluster and form ordered assemblies that restrain the lipids from diffusing freely. This lateral lipid inhomogeneity was first discovered in the Golgi apparatus and termed lipid raft (Simons and van Meer, 1988). Lipid rafts were defined as small, 10-200 nanometer-sized microdomains enriched in sphingolipids and sterols, and formed by lipid-lipid interactions (Figure 3). The molecular structure of sphingolipids, especially those featuring very long and saturated fatty acid chains, is assumed to facilitate the separation from glycerophospholipids. Owing to the particular length of the fatty acids, lipid rafts were proposed to be thicker than the remaining membrane (Gandhavadi *et al.*, 2002), hence may recruit a special subset of transmembrane proteins with longer membrane-spanning helices. Certain protein modifications, for instance a GPI-anchor or a palmitoyl group, preferentially associate with and target the proteins to lipid rafts (Brown and Rose, 1992; Zacharias *et al.*, 2002; Foster *et al.*, 2003).

Despite decades of intensive research, the lipid raft hypothesis remains highly controversial mainly due to the lack of appropriate techniques to study them in live cells (Munro, 2003; Jacobson *et al.*, 2007; Lingwood and Simons, 2010). Fluorescence microscopy is still one of the best methods to analyze biological significant microdomains. Recent developments in high-resolution microscopy and single molecule tracking even allow for the examination of very small (less than 50 nanometer) and/or very short-lived nanoclusters (Eggeling *et al.*, 2009). In contrast, most biophysical studies of lipid rafts were conducted on synthetic membranes that have considerable drawbacks. Artificial membranes neither reflect the complex lipid composition of biological membranes nor feature their high and diverse protein content. Both lipid and protein composition tremendously influence the lateral membrane organization of living cells. Moreover, the physical behavior of lipids in membranes depends on the applied conditions. Thus, *in vitro* membrane reconstitution assays serve well to examine basic principles of lipid behavior but are less suitable to prove the existence of lipid rafts *in vivo*.

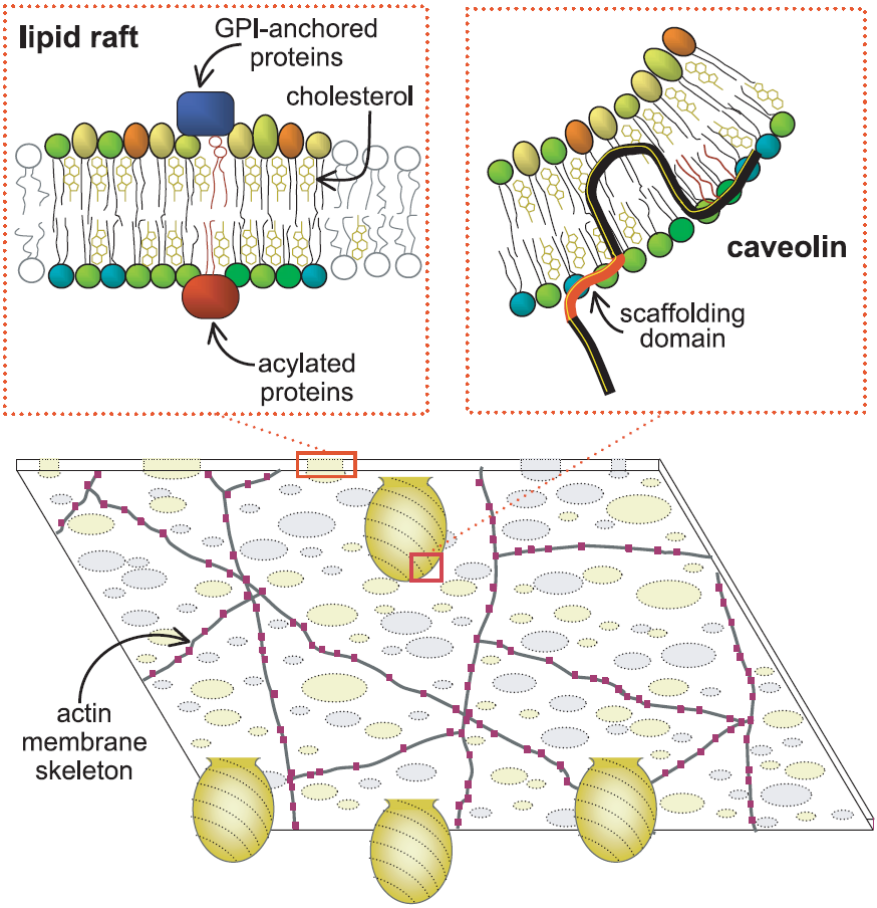

Figure 3: The lateral organization of the plasma membrane. Lipid rafts (top, left) and caveolae (top, right) are plasma membrane microdomains specifically enriched in sphingolipids and cholesterol. The cortical actin cytoskeleton interacts with membrane-anchored proteins (bottom), thus fencing larger membrane compartments and restricting the diffusion of molecules within such domains. (Picture taken from (Laude and Prior, 2004).)

One prominent and well studied example of raft-like microdomains are caveolae, as they are easily detectable by electron or fluorescence microscopy (Figure 3). Caveolae are protein-coated, flask-like membrane invaginations with diameters of about 50-100 nanometers. Their major structural components are caveolins. Caveolins are small proteins that are partially integrated in the plasma membrane where they interact with fatty acids and cholesterol (Murata *et al.*, 1995). The ability of caveolins to form oligomeric assemblies leads to local protein-driven enrichment of sphingolipids and cholesterol (Monier *et al.*, 1996). Caveolae appear quite stable and immobile under normal conditions, but their formation is dynamic and regulated by kinases and caveolin-interacting proteins (Pelkmans and Zerial, 2005; Hill *et al.*,

2008; Liu *et al.*, 2008; Liu and Pilch, 2008). Caveolin-coated membranes are able to pinch off the plasma membrane, forming caveolin-containing vesicles. These vesicular carriers may represent a distinct endocytic pathway involved in fatty acid transport (Meshulam *et al.*, 2006; Meshulam *et al.*, 2011).

The most prominent function proposed for caveolae - and lipid raft microdomains in general - is their role in signaling. The particular lipid composition of these microdomains accounts for the recruitment and local clustering of signaling molecules, thus providing a platform for efficient and specific signal transduction (Bastiani and Parton, 2010). In addition, the scaffolding domain of caveolin binds a variety of signaling proteins, such as G-protein-coupled or growth-factor receptors (Couet *et al.*, 1997). Caveolae might also act as mechanosensors and membrane reservoirs (Boyd *et al.*, 2003; Rizzo *et al.*, 2003; Sinha *et al.*, 2011). In times of acute membrane stress, for instance during osmotic swelling, the flask-shaped invaginations may provide the required extra membrane to rapidly counteract surface tension (Sinha *et al.*, 2011). Furthermore, ion channels cluster and are gated at caveolae. Hence, changes in the membrane structure upon increased tension could be one mechanism to activate these channels (Harvey and Calaghan, 2012).

Large plasma membrane domains

The individual movement of proteins and lipids is not only restricted within raft-like microdomains, but also within larger forms of plasma membrane compartments. The dimensions of such macrodomains range from several hundred nanometers to some micrometers. Macrodomains can emerge from the coalescence of several lipid raft microdomains mediated by protein-protein interactions (Gupta and DeFranco, 2003). Alternatively, transmembrane proteins are anchored to the cortical cytoskeleton or to the extracellular matrix (or the cell wall in yeast) and may form a lateral, fence-like diffusion-barrier for the encircled lipids and proteins (Kusumi *et al.*, 1993; Sako and Kusumi, 1994). Molecules within such compartments can freely diffuse but are locally restricted by the fences for a certain time (Figure 3). Single particle tracking revealed that proteins and lipids are able to exit into adjacent compartments through dynamic gaps between the protein barriers, an occurrence referred to as “hop-diffusion” (Fujiwara *et al.*, 2002).

In polarized epithelial cells, for instance Madin-Darby canine kidney (MDCK) cells, the composition of the basolateral plasma membrane significantly differs from the apical membrane. The apical membrane is particularly robust as it is exposed to the external environment. To achieve this robustness the apical plasma membrane is specifically enriched in glycosphingolipids and cholesterols. These lipids are clustered in the Golgi apparatus and subsequently directed to the apical surface (Schuck and Simons, 2004; Rodriguez-Boulan and Musch, 2005). Lateral tight junctions act as diffusion barriers to maintain cell polarity by preventing the exchange of protein and lipids between the two compartments (Rodriguez-Boulan and Nelson, 1989; Balda *et al.*, 1996).

The plasma membrane organization in Saccharomyces cerevisiae

Technical advances, particularly in light microscopy, allowed for rapid progress in the study of fungal plasma membrane compartmentalization. The plasma membrane of the budding yeast *S. cerevisiae*, for instance, is also organized into macro- and microdomains. In dividing cells, septin proteins act as diffusion barriers at the neck between the growing bud and the mother cell, therefore creating two separated plasma membrane macrodomains with different protein compositions (Barral *et al.*, 2000; Takizawa *et al.*, 2000). In addition, work from Malinska *et al.* revealed the existence of two exclusive plasma membrane compartments named after prominent proteins localizing to them (Malinska *et al.*, 2003; Malinska *et al.*, 2004). The membrane compartment occupied by Can1 (MCC) comprises a number of immobile microdomains, surrounded by the dynamic, network-like membrane compartment occupied by Pma1 (MCP) (Figure 4). Pma1 is a highly abundant proton ATPase that requires sphingolipids with very long fatty acids, but not sterols, for its sorting into rafts in the endoplasmatic reticulum (ER) and its subsequent transport to the plasma membrane (Lee *et al.*, 2002; Gaigg *et al.*, 2005; Gaigg *et al.*, 2006).

The MCC is by far better characterized than the MCP. Originally defined by the localization of the green fluorescent protein (GFP)-tagged arginine permease Can1 into extremely stable, punctuate patches of about 300 nanometers, it was soon discovered that also other transmembrane proteins localize to these microdomains. The Sur7 family, Nce102 and Fhn1 (all of them tetra-spanning membrane proteins), as well as other amino acid transporters, such as the uracil permease Fur4, were all found to colocalize with Can1 in MCCs (Malinska *et al.*, 2003;

Malinska *et al.*, 2004). Further work revealed that the proteins Pil1 and Lsp1 form protein assemblies at the cytosolic leaflet of the plasma membrane underlying the MCC, and these stable assemblies were termed eisosomes (Walther *et al.*, 2006). Pil1 is responsible for the uniform distribution of MCC/eisosomes around the plasma membrane, as the peculiar domain pattern collapses into very few remnants in *pil1Δ* mutant cells. These remnants form at huge plasma membrane invaginations and the remaining MCC-proteins concentrate there. Lsp1, though sharing high sequence similarity with Pil1, is dispensable for eisosomal assembly and MCC formation (Walther *et al.*, 2006). Pil1 not only regulates the pattern, but also determines the number (about 30 per cell) and size (approximately 3000 Pil1 molecules per eisosome) of eisosomes (Moreira *et al.*, 2009). The expression of both Pil1 and Lsp1 is cell cycle regulated. Eisosome assembly starts at the neck of growing yeast buds and proceeds one after the other towards the bud tip (Moreira *et al.*, 2009).

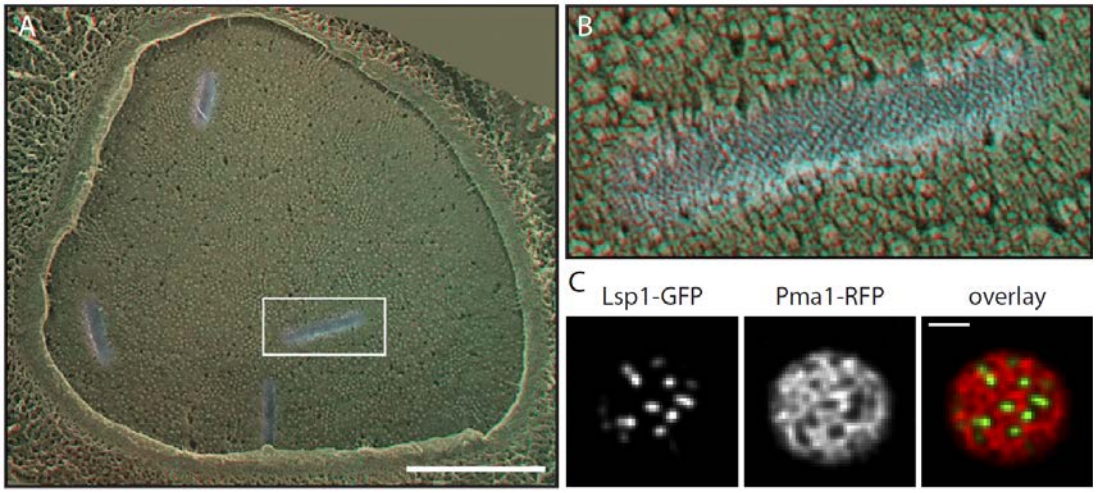

Figure 4: The yeast plasma membrane is organized into two mutually exclusive domains. A-B) Electron microscopic images of the yeast plasma membrane. The magnification (B) reveals the furrow-like invagination of the MCC. Scale bar 500 nm. C) Fluorescence microscopic optical top sections of a yeast cell demonstrating that the MCC, depicted by Lsp1-GFP (left, green), and the MCP, depicted by Pma1-RFP (middle, red), are mutually exclusive plasma membrane domains. Scale bar 1 µm. (Pictures A and B taken from (Karotki *et al.*, 2011).)

Recently, eisosomes were analyzed at an ultrastructural level using freeze-fracture and cryo-electron microscopy, as well as crystallography. These studies revealed that MCCs are long (approx. 300 nanometers), furrow-like membrane invaginations of about 50 nanometers width (Figure 4), and that eisosomal proteins coat these furrows (Stradalova *et al.*, 2009; Karotki *et al.*,

2011). The crystal structure of a Lsp1-construct shows that Pil1 and Lsp1 are bin-amphiphysin-rvs (BAR) domain containing proteins (Ziolkowska *et al.*, 2011). BAR-domain proteins bind to membranes and induce local curvature (Frost *et al.*, 2009). As predicted from their structure, Pil1 and Lsp1 are able to bind to liposomes, preferentially those enriched in phosphoinositides (Karotki *et al.*, 2011). The lipid binding together with protein self-assembly induces liposome tubulation. Thus, it is likely that Pil1 and Lsp1 are not only inducing the furrow-like invaginations *in vivo*, but may also cluster specific plasma membrane lipids, such as PtdIns(4,5)P_2 into these domains. Grossmann *et al.* suggested that MCCs are enriched in ergosterol, since filipin, a fluorescent sterol-binding macrolide, colocalizes with an MCC marker protein (Grossmann *et al.*, 2007). However, the possibilities to directly visualize the distribution of sterols (and sphingolipids) in living cells are limited. The existing lipid stains, such as filipin, are prone to create artifacts, thus are less suitable to determine the lipid composition of plasma membrane domains.

Sites of clathrin-mediated endocytosis may represent a third exclusive plasma membrane compartment in yeast, since typical endocytic markers do not colocalize with MCCs but are predominantly concentrated to the bud tip (Kaksonen *et al.*, 2005; Brach *et al.*, 2011). In addition, these endocytic sites appear ultra-structurally as thin, finger-like membrane invaginations (Mulholland *et al.*, 1994), hence they are clearly distinguishable from the large furrows of MCCs. Clathrin-mediated endocytosis is well characterized in yeast. Numerous studies contributed to our current knowledge of the sequential recruitment of protein modules to endocytic sites, leading to actin patch assembly and membrane internalization (Kaksonen *et al.*, 2005; Kaksonen *et al.*, 2006; Toret and Drubin, 2006).

Despite substantial progress in the characterization of plasma membrane compartments, it remains unclear how many distinct domains exist at any given time point and what their function is. In addition, the formation of microdomains is difficult to determine. Do lipids cluster to provide small raft-like platforms for the subsequent recruitment of proteins or do proteins induce lipid clustering through binding to lipids and interacting with other lipid-binding proteins? It is very likely that both, lipid connectivity and protein scaffolds play a concomitant role in the organization of membranes.

The synthesis and regulation of sphingolipids in yeast

The biosynthetic pathway of yeast sphingolipids

Complex sphingolipids are essential components of plasma membranes and specifically enriched there. In yeast, they comprise more than 30 mol percent of all plasma membrane lipids and predominantly reside in the outer membrane leaflet. Complex sphingolipids consist of a sphingoid long chain base (dihydrosphingosine (DHS) or phytosphingosine (PHS)), a long chain fatty acid and a hydrophilic sugar head group. The main organelle for the synthesis of ceramide, as well as most glycerolipids and sterols, is the ER.

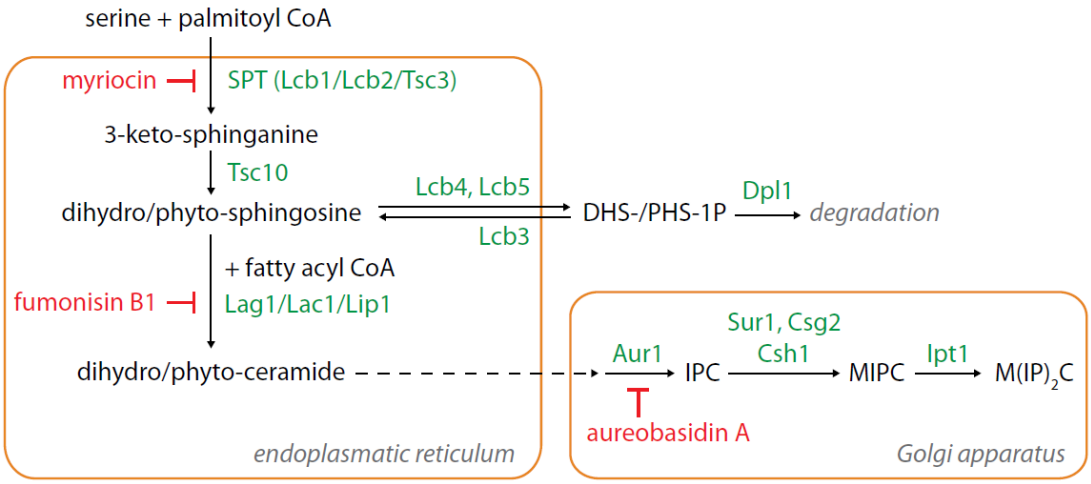

Figure 5: Simplified scheme of the biosynthetic pathway of sphingolipids in *S. cerevisiae*. Major lipid metabolites (black), enzymes (green) and enzyme-inhibiting antifungal toxins (red) are shown. SPT: serine palmitoyl transferase; DHS-/PHS-1P: dihydro-/phyto-sphingosine 1-phosphate; IPC: inositolphosphoceramide; MIPC: mannose-IPC; $M(IP)_2C$: mannose-di-IPC.

The very first step of sphingolipid synthesis is the condensation of cytoplasmic serine and palmitoyl CoA into 3-keto-sphinganine (Figure 5). This reaction is catalyzed by the serine palmitoyl transferase (SPT) complex, consisting of an Lcb1-Lcb2 heterodimer (Hanada, 2003) and a third protein, Tsc3. Tsc3 is required for SPT function under elevated temperatures but its exact role is not known. The intermediate 3-keto-sphinganine is subsequently reduced to DHS (or sphinganine) by the reductase Tsc10 in the cytoplasmic leaflet of the ER membrane. The hydroxylase Sur2 converts DHS to PHS. Both sphingoid bases can exit the sphingolipid synthesis pathway and are phosphorylated by the sphingosine kinases Lcb4 and Lcb5 (Nagiec *et al.*, 1998).

The resulting DHS/PHS 1-phosphates promote heat stress resistance, and PHS 1-phosphate was recently shown to regulate the expression of respiratory genes by activating specific transcription factors (Cowart *et al.*, 2010). The phosphate-lyase Dpl1 degrades DHS/PHS 1-phosphates to ethanolamine phosphate and fatty aldehydes. Alternatively, the phosphatase Lcb3 hydrolyzes DHS/PHS 1-phosphates to sphingoid bases (Cowart and Obeid, 2007). Long chain bases remaining in the ER are condensed with fatty acyl CoA to yield dihydro- or phytoceramide. The ceramide synthase complex required for this reaction consists of Lag1, Lac1 and Lip1. Ceramide is transported from the ER to the Golgi apparatus for further processing. Alternatively, a protein termed CERT in mammalian cells is able to catalyze inter-membrane, non-vesicular transfer of ceramide from the ER to the Golgi (Hanada *et al.*, 2009). Though ER-Golgi contact sites are important for non-vesicular ceramide trafficking in yeast, a fungal ortholog to CERT remains to be identified (Funato and Riezman, 2001).

In the Golgi apparatus, polar head groups are attached to the ceramide backbone resulting in complex sphingolipids. The first intermediate is inositolphosphoceramide (IPC), generated through the addition of inositol to dihydro- or phytoceramide by the IPC synthase Aur1 (Nagiec *et al.*, 1997). The next step, the transfer of mannose to the inositol group of IPC to yield mannose-IPC (MIPC), is catalyzed by the mannosyltransferase complex consisting of the catalytic subunit Csg1 (Sur1) or Csh1, and the regulatory subunit Csg2 (Uemura *et al.*, 2003). Finally, MIPC is converted to mannose-di-inositolphosphoceramide ($M(IP)_2C$) through the attachment of a second inositol phosphate emanating from PtdIns, a reaction that requires Ipt1 (Dickson *et al.*, 1997b). The attachment of head groups to the ceramide backbone takes place at the luminal sides of the Golgi membranes, therefore complex sphingolipids - once transported via the secretory pathway to the plasma membrane - predominantly locate to the outer membrane leaflet.

Antifungal toxins inhibit various steps in the sphingolipid pathway, therefore depleting certain pools of metabolites (Figure 5). Myriocin, for instance, inhibits SPT, thus blocking the very first step of sphingolipid synthesis (Fujita *et al.*, 1994; Sun *et al.*, 2000). Aureobasidin A inhibits Aur1, resulting in a block of complex sphingolipid synthesis and a concomitant accumulation of long chain bases (Nagiec *et al.*, 1997). Pharmacological inhibition of enzymes catalyzing sphingolipid

synthesis remains one of the most useful experimental tools to dissect the signaling pathways downstream of sphingolipid metabolites or their regulation.

The function and regulation of sphingolipids

Sphingolipids play significant roles in the regulation of diverse cellular processes and act either directly as intracellular signaling molecules or indirectly as major structural components of (plasma) membranes. Ceramides and complex sphingolipids are required for the trafficking of membrane proteins, such as Pma1, to the plasma membrane (Bagnat *et al.*, 2001; Lee *et al.*, 2002). Likewise, sphingolipids are required for the stable association of GPI-anchored proteins at the plasma membrane (Dickson *et al.*, 2006). Moreover, the physical properties of membranes, such as fluidity and thickness, are altered through the incorporation of sphingolipids. Additionally, ceramide and sphingosine are "bioactive" metabolites and function as second messengers. In yeast, they are involved in the regulation of cell growth, cell wall synthesis and heat stress resistance (Cowart and Obeid, 2007). Sphingoid bases were implicated in the regulation of the actin cytoskeleton and endocytosis by directly activating protein kinases. At least *in vitro*, PHS stimulates the activity of AGC (protein kinase A, G and C) kinases (Friant *et al.*, 2001; Liu *et al.*, 2005). Studies in mammalian cells revealed that sphingolipids are involved in many common and fatal human diseases such as diabetes, Alzheimer's disease and cancer, but the direct targets of long chain bases or sphingolipids are not known (Cowart and Obeid, 2007).

Despite our thorough understanding of most of the sphingolipid-anabolizing or degrading enzymes, the knowledge about the regulation of these enzymes is lacking behind. How is the abundance of certain bioactive metabolites regulated in relation to other lipid species or according to cellular requirements? The compartmentalization of ceramide synthesis in the ER and complex sphingolipid synthesis in the Golgi apparatus could provide one mechanism to shift the balance between distinct lipid species, for instance by altering the activity of the responsible ceramide transfer protein CERT in mammalian cells.

In yeast, long chain base synthesis, in particular C_{20}-DHS and C_{20}-PHS, is rapidly (within minutes), but only transiently, induced upon heat stress as a thermo-protective response and leads to cell cycle arrest (Dickson *et al.*, 1997a; Meier *et al.*, 2006). Since no intracellular stores of sphingolipids are present and only little transcriptional regulation of the sphingolipid-

anabolizing enzymes was observed, sphingolipid levels are most likely upregulated by increased enzyme activity, for instance caused by posttranslational modifications. In addition to *de novo* synthesis, ceramides can also be generated through hydrolysis of the polar head group of complex sphingolipids by the IPC phospholipase C, Isc1. In both cases, the relevant upstream proteins, which alter the enzyme activity of sphingolipid synthases or hydrolases, remain ill defined. It is also not known how a cell perceives sphingolipid levels and how it maintains sphingolipid homeostasis during cell growth or upon changes in environmental conditions.

The role of TOR and Ypk kinases in sphingolipid signaling

In order to screen for proteins involved in sphingolipid signaling, mRNA microarrays were used to compare protein expression levels under different conditions, for instance during heat shock or in mutants with defective SPT. One of these screens revealed altered levels of Tor2 (Beeler *et al.*, 1998), a conserved serine/threonine kinase known to be an important regulator of cell growth (De Virgilio and Loewith, 2006). TOR (target of rapamycin) kinase assembles into two structurally and functionally distinct multi-protein complexes termed TOR kinase complex 1 (TORC1) and TOR kinase complex 2 (TORC2) (Loewith *et al.*, 2002). Yeast cells have two TOR kinases, Tor1 and Tor2. While both kinases can be a component of TORC1, Tor2 is the exclusive kinase in TORC2. The two complexes further consist of one shared (Lst8) and several unique components (Kog1 and Tco89 in TORC1; Avo1, Avo2, Avo3 and Bit61 in TORC2) (Loewith *et al.*, 2002; Wullschleger *et al.*, 2005). Though initially identified in yeast, the two complexes are structurally and functionally conserved. Orthologs of their components are so far characterized in many other organisms ranging in evolutionary complexity from fungi to human (Jacinto *et al.*, 2004; Sarbassov *et al.*, 2004).

The two TOR complexes regulate different aspects of cell growth. The specific inhibition of TORC1 by rapamycin facilitated the analysis of its function and downstream substrates. TORC1 controls the temporal aspect of cell proliferation in response to environmental cues (Martin and Hall, 2005). When growth conditions are favorable, for instance in the presence of nutrients or energy, TORC1 stimulates anabolic processes, such as ribosome biogenesis and protein synthesis, and concomitantly blocks catabolic processes, for instance autophagy. In contrast to TORC1, TORC2 is insensitive to rapamycin. The lack of a TORC2-specific inhibitor hampered the characterization of processes regulated by TORC2 (Loewith *et al.*, 2002). One

established role of TORC2, however, is the spatial regulation of cell growth. In yeast, TORC2 controls cell polarity through the organization of the actin cytoskeleton (Figure 6). Furthermore, TORC2 and its downstream substrate Ypk1 kinase are required for the efficient internalization of plasma membrane receptors and fluid-phase endocytosis (deHart *et al.*, 2002; deHart *et al.*, 2003).

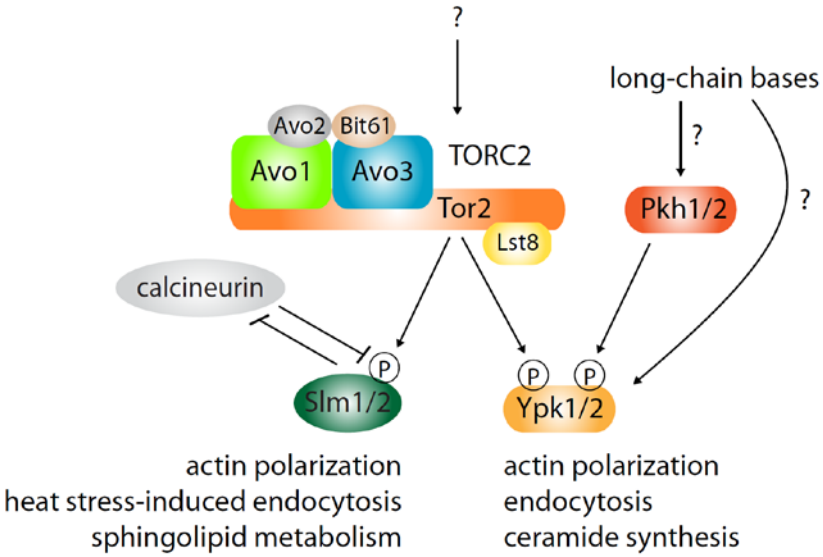

Figure 6: Cartoon illustrating the signaling network of TORC2 and Pkh kinases. TORC2, Pkh kinases and their downstream targets Ypk kinases and Slm proteins regulate actin polarization, endocytosis and sphingolipid metabolism.

Ypk1 and Ypk2 are functionally redundant kinases and orthologs of mammalian serum and glucocorticoid-inducible kinase (SGK) (Casamayor *et al.*, 1999). They belong to the highly conserved family of AGC kinases (Jacinto and Lorberg, 2008). Full activation of AGC family kinases requires phosphorylation of two conserved sites. The PDK1 site in the catalytic domain of AGC kinases is phosphorylated by phosphoinositide dependant kinase 1 (PDK1), while the so-called PDK2 sites in the hydrophobic and turn motif are phosphorylated by TOR kinase (Casamayor *et al.*, 1999; Jacinto and Lorberg, 2008). Yeast TORC2 phosphorylates both the hydrophobic and turn motif of Ypk2 (Kamada *et al.*, 2005). Ypk2 was identified as multi-copy suppressor of the *tor2* deletion defect, and the constitutively active allele $YPK2^{D239A}$ suppresses the lethality caused by a *tor2* deletion or a mutation in *TOR2* that disrupts its kinase activity (Kamada *et al.*, 2005). The PDK1 site of Ypk kinases is phosphorylated by the yeast orthologs of PDK1, the redundant kinases Pkh1 and Pkh2. Pkh kinase activity is required for the Ypk1-mediated regulation of ubiquitin-dependant receptor internalization (deHart *et al.*, 2002). Pkh1

and Pkh2 localize to the plasma membrane, but since they – unlike their mammalian ortholog PDK1 – lack a PtdIns(4,5)P_2-binding PH-domain, their recruitment mechanism remains to be determined (Roelants *et al.*, 2002).

Both, Pkh and Ypk kinases, are functionally linked to sphingolipid metabolism. Ypk1, for instance, localizes to the cytoplasm and the plasma membrane, and the latter localization seems to be dependent on sphingolipid levels (Sun *et al.*, 2000; Roelants *et al.*, 2002). The Riezman laboratory revealed that overexpression of Pkh kinases bypasses the requirement of sphingoid base synthesis for endocytosis and for proper organization of the actin cytoskeleton (Zanolari *et al.*, 2000; Friant *et al.*, 2001). Consistently, sphingolipids seem to play a direct role in the upstream regulation of the kinases since the long chain base PHS induces auto-phosphorylation and activation of the AGC kinases Ypk1/2 and Pkh1/2 *in vitro* (Liu *et al.*, 2005). Moreover, Pkh and Ypk kinases are multicopy suppressors of lethality caused by the sphingolipid biosynthesis inhibitor myriocin (Sun *et al.*, 2000). Both Ypk1 phosphorylation at the hydrophobic motif (PDK2 site) and Ypk1 kinase activity are required for myriocin resistance, suggesting also a role for TORC2 in sphingolipid metabolism (Tanoue *et al.*, 2005).

Besides *TOR2*, also the TORC2-specific component *AVO3* was identified as temperature-sensitive suppressor of the lethality of a *CSG2* deletion (Beeler *et al.*, 1998). Deletion of *CSG2* results in IPC accumulation, which is toxic in the presence of calcium. Hence, suppressor mutations were mostly found in genes coding for enzymes directly involved in sphingolipid synthesis. For instance, mutations that disrupt the function of the 3-keto-sphinganine reductase Tsc10 result in decreased amounts of IPC. The identification of suppressor mutations in *TOR2* and *AVO3* suggested that TORC2 modulates sphingolipid synthesis. Indeed, TORC2 activity is required for ceramide synthesis (Aronova *et al.*, 2008). A mutant allele of *AVO3* (*avo3-30*) causes decreased levels of phytoceramide and hypo-phosphorylation of Ypk2. Expression of the constitutively active allele $YPK2^{D239A}$ restores phytoceramide levels, indicating that TORC2 via Ypk2 positively regulates ceramide synthase (Aronova *et al.*, 2008).

In addition, TORC2 phosphorylates two other proteins, Slm1 and Slm2, which were linked to sphingolipid metabolism (Audhya *et al.*, 2004; Fadri *et al.*, 2005; Tabuchi *et al.*, 2006). The essential Slm proteins are functionally redundant and possess a phosphoinositide-binding PH-domain, which mediates their plasma membrane localization (Audhya *et al.*, 2004; Fadri *et al.*,

2005). Slm proteins are involved in the regulation of actin polarization downstream of PtdIns(4,5)P_2 and TORC2 (Audhya *et al.*, 2004; Fadri *et al.*, 2005). However, the actin organization defect of *slm* mutant cells can be rescued by disruption of Isc1, a phospholipase that catabolizes complex sphingolipids back to ceramide, suggesting that Slm proteins either negatively regulate Isc1 or play a positive role in sphingolipid synthesis (Tabuchi *et al.*, 2006). Consistently, Slm protein function is required for growth in the presence of myriocin and for sphingolipid-dependant processes, such as the heat stress-induced endocytosis of the uracil permease Fur4 (Bultynck *et al.*, 2006). Heat stress induces Slm protein phosphorylation, which is required for cell survival and dependent on sphingolipid levels and Pkh kinase activity (Daquinag *et al.*, 2007). The Ca^{2+}/calmodulin-regulated protein phosphatase calcineurin antagonizes Slm protein phosphorylation (Bultynck *et al.*, 2006; Tabuchi *et al.*, 2006). Inhibition of calcineurin results in enhanced interaction between TORC2 and Slm proteins, and hyper-phosphorylation of the latter (Mulet *et al.*, 2006). Thus, calcineurin is a negative regulator of TORC2 activity and sphingolipid synthesis (Mulet *et al.*, 2006; Tabuchi *et al.*, 2006). In support of this model, the diminished phytoceramide levels caused by the *avo3-30* mutant were restored through the deletion of calcineurin (Aronova *et al.*, 2008).

Together, these studies suggest that Ypk kinases and Slm proteins integrate signals from TORC2 and Pkh kinases to control actin polarization and sphingolipid metabolism. However, physiological regulators of TORC2 and Pkh kinases, as well as downstream targets of Ypk kinases, remain elusive. It is also not known how Slm proteins couple phosphoinositide and sphingolipid signaling and how they mechanistically regulate downstream processes.

Aims

The plasma membrane is subject to constant remodeling. During cell growth or in a hypo-osmotic environment, for instance, the increase in cell volume requires a concomitant expansion of the plasma membrane. Complex sphingolipids are essential components of the plasma membrane and perform, besides their structural role, important regulatory functions. While the synthesis of sphingolipids is well understood, only little is known about how their

levels are regulated, and how the cell perceives alterations of complex sphingolipids in the plasma membrane.

Previous studies linked the TORC2-Pkh-Ypk kinase network to the regulation of sphingolipid metabolism. Specifically, TORC2 activates Ypk2 kinase to positively regulate the synthesis of ceramides, precursors of complex sphingolipids (Aronova *et al.*, 2008). In addition, TORC2 phosphorylates the essential Slm proteins, which are linked to sphingolipid metabolism (Tabuchi *et al.*, 2006; Daquinag *et al.*, 2007). However, it was not known what regulates TORC2 and whether TORC2 responds to altered complex sphingolipid levels in the plasma membrane. Subcellular fractionation, immunogold electron microscopy and immunofluorescence studies revealed that Tor2 kinase partially associates with the plasma membrane (Kunz *et al.*, 2000; Wedaman *et al.*, 2003; Aronova *et al.*, 2007) but the *in vivo* localization of TORC2 was not known. Thus, we first set out to analyze the subcellular localization of TORC2 by live cell microscopy. We discovered that TORC2 localizes exclusively to the plasma membrane and that this association is essential for viability. Based on these findings, we hypothesized that TORC2 perceives altered complex sphingolipid levels in the plasma membrane and thereupon activates its downstream effectors, Ypk kinases and Slm proteins, which also reside at the plasma membrane (Sun *et al.*, 2000; Audhya *et al.*, 2004). To test this hypothesis, we were pursuing three aims. Aim 1: Determine whether TORC2 activity is regulated by altered sphingolipid levels. Aim 2: Elucidate how TORC2 perceives complex sphingolipid levels. Aim 3: Characterize the role of Slm proteins in relation to Ypk kinase function in the regulation of sphingolipid levels.

The outcome of this thesis will reveal upstream regulatory factors of TORC2 and help to understand the mechanism of TORC2 activation. The results will provide new insights into how cells perceive the lipid composition of the plasma membrane and how cells maintain homeostasis of complex sphingolipids.

Abstracts of publications

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Publication I

TORC2 plasma membrane localization is essential for cell viability and restricted to a distinct domain

Berchtold, D. and T. C. Walther

Mol Biol Cell 20(5): 1565-1575 (2009)

Abstract

The conserved target of rapamycin (TOR) kinases regulate many aspects of cellular physiology. They exist in two distinct complexes, termed TOR complex 1 (TORC1) and TOR complex 2 (TORC2), that posses both overlapping and distinct components. TORC1 and TORC2 respond differently to the drug rapamycin and have different cellular functions: whereas the rapamycin-sensitive TORC1 controls many aspects of cell growth and has been characterized in great detail, the TOR complex 2 is less understood and regulates actin polymerization, cell polarity, and ceramide metabolism. How signaling specificity and discrimination between different input signals for the two kinase complexes is achieved is not understood. Here, we show that TORC1 and TORC2 have different localizations in Saccharomyces cerevisiae. TORC1 is localized exclusively to the vacuolar membrane, whereas TORC2 is localized dynamically in a previously unrecognized plasma membrane domain, which we term membrane compartment containing TORC2 (MCT). We find that plasma membrane localization of TORC2 is essential for viability and mediated by lipid binding of the C-terminal domain of the Avo1 subunit. From these data, we suggest that the TOR complexes are spatially separated to determine downstream signaling specificity and their responsiveness to different inputs.

Full text (pdf)

Publication II

Plasma membrane stress induces relocalization of Slm proteins and activation of TORC2 to promote sphingolipid synthesis

Berchtold, D., M. Piccolis, N. Chiaruttini, I. Riezman, H. Riezman, A. Roux, T.C. Walther, and R. Loewith

Nat Cell Biol. 14: 542–547 (2012).

Abstract

The plasma membrane delimits the cell, and its integrity is essential for cell survival. Lipids and proteins form domains of distinct composition within the plasma membrane. How changes in plasma membrane composition are perceived, and how the abundance of lipids in the plasma membrane is regulated to balance changing needs remains largely unknown. Here, we show that the Slm1/2 paralogues and the target of rapamycin kinase complex 2 (TORC2) play a central role in this regulation. Membrane stress, induced by either inhibition of sphingolipid metabolism or by mechanically stretching the plasma membrane, redistributes Slm proteins between distinct plasma membrane domains. This increases Slm protein association with and activation of TORC2, which is restricted to the domain known as the membrane compartment containing TORC2 (MCT). As TORC2 regulates sphingolipid metabolism, our discoveries reveal a homeostasis mechanism in which TORC2 responds to plasma membrane stress to mediate compensatory changes in cellular lipid synthesis and hence modulates the composition of the plasma membrane. The components of this pathway and their involvement in signalling after membrane stretch are evolutionarily conserved.

Full text (pdf)

Discussion

The spatial organization of proteins into distinct plasma membrane compartments provides signaling specificity

The spatial separation of TORC1 and TORC2 facilitates differential regulation

The plasma membrane is the barrier that separates the cellular interior from its environment. Likewise, membranes enclose intracellular organelles to separate them from each other and the cytoplasm, thereby creating specialized subunits with particular functions. Membranes from different organelles have distinguishable lipid and protein compositions. The resulting subcellular compartments facilitate the control of signaling events by keeping proteins separated to prevent inadvertent activation, or by locally concentrating proteins to increase the efficiency of signal transduction.

In this study, we found two major regulators of cell growth, TORC1 and TORC2, spatially separated within the cell. While TORC1 localizes to the vacuolar membrane, we detected TORC2 exclusively at the plasma membrane (Figure 7).

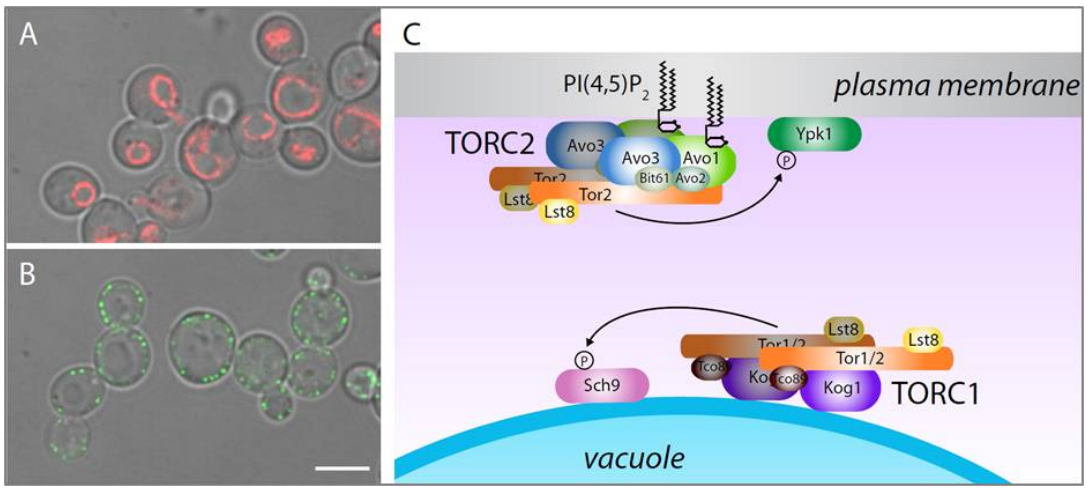

Figure 7: TORC1 and TORC2 are spatially separated. A) Kog1-GFP (red), a specific component of TORC1, localizes to the vacuolar membrane. B) Avo3-GFP (green), a unique TORC2 component, forms foci at the plasma membrane. Scale bar 5 µm. C) Cartoon illustrating the spatial separation of TORC1 and TORC2 as well as their specific downstream kinases Sch9 and Ypk1. Dimerization of either complex is indicated. The PH-domain of Avo1 binds to the plasma membrane-specific lipid PtdIns(4,5)P_2.

How are the two TOR complexes recruited to their specific compartments? The different cellular localizations are most likely attributed to the unique subunits of either complex. Avo1, a specific and essential component of TORC2, features a lipid-binding PH-domain, which exhibits specific affinity to PtdIns(4,5)P_2 and is essential for TORC2 plasma membrane localization. TORC1 recruitment to the vacuolar membrane may likewise be mediated by a specific (and essential) subunit, such as Kog1, but the targeting mechanism remains to be further investigated.

TORC1 and TORC2 share the same kinase, but their spatial separation and their specific components account for a different signaling output of TOR kinase. In addition, the segregation of the two complexes enables TOR kinase to respond to different upstream stimuli. Sch9 kinase, for instance, is specifically phosphorylated by TORC1 when nutrients, such as amino acids or nitrogen, are available (Urban *et al.*, 2007; Binda *et al.*, 2009). Consistently, Sch9 localizes exclusively to the vacuolar surface, hence, in close proximity to TORC1 (Jorgensen *et al.*, 2004; Urban *et al.*, 2007). In contrast, Ypk1, which localizes to the cytoplasm and the plasma membrane (Sun *et al.*, 2000; Roelants *et al.*, 2002), is specifically phosphorylated by TORC2 upon plasma membrane stress (Figure 7).

Despite intensive investigation of the regulation of TOR complexes, mechanistic details of their activation remain poorly defined. TORC1 and TORC2 localize constitutively to the vacuolar and plasma membrane, respectively, meaning that different conditions, which alter TOR kinase activity, have no effect on the localization of the complexes (Binda *et al.*, 2009). In turn, this suggests that altered subcellular localization of the TOR kinase complexes is not the mechanism that regulates their activity towards downstream effectors. The activation of TORC1 and TORC2 may be regulated indirectly through the dynamic recruitment of substrates and/or adaptor proteins, which are responsive to upstream signals. For instance, subunits of the EGO complex interact with TORC1 at the vacuolar membrane and this interaction facilitates Sch9 activation. Leucine starvation disrupts the association between the EGO complex and TORC1, leading to decreased phosphorylation of Sch9 (Binda *et al.*, 2009). Likewise, the interaction of Slm proteins with TORC2 facilitates the recruitment and activation of Ypk1. Slm proteins hence act as adaptors, which are responsive to plasma membrane stress and communicate it to TORC2 through binding to a specific TORC2 subunit, for instance Avo1 or Avo3.

TORC2 is restricted to a small and distinct plasma membrane compartment

TORC2 is not evenly distributed in the plane of the plasma membrane, but is laterally restricted to a distinct domain, the membrane compartment containing TORC2 (MCT). The organization of the plasma membrane into distinct microdomains additionally helps to prevent unspecific signaling. Potential downstream effectors of TORC2 have to be in close proximity to the kinase for efficient signal transduction. How are proteins targeted to the MCT?

The only identified proteins localizing to the MCT are the components of TORC2, the Slm proteins and Ypk1 kinase. TORC2 and Slm proteins are anchored to the plasma membrane via their PtdIns(4,5)P_2-binding PH-domain. The PH-domain of Avo1 and Slm1 is required for their plasma membrane targeting, but is not sufficient to specifically localize the proteins to the MCT. First, Slm proteins do not exclusively localize to the MCT but also reside at eisosomes. Second, expression of the GFP-tagged PH-domain of either Slm1 or Avo1 exhibits an evenly distributed signal along the plasma membrane without foci formation (unpublished data of D. Berchtold). Third, the replacement of the Avo1 PH-domain with a CAAX-motif does not disrupt the formation of cortical TORC2 foci. Thus, a secondary signal, for instance the ability to bind to MCT-specific lipids or to associate with (a) yet unidentified scaffold protein(s) in the MCT, is required to target proteins to the MCT.

The MCT, the MCP and eisosomes are the only mutually exclusive plasma membrane compartments described so far in yeast and together cover nearly the entire cell surface. This finding, however, does not imply that the plasma membrane is organized into a limited number of distinct domains and that all plasma membrane proteins can be simply assigned to one or two non-overlapping membrane compartments. A recent study demonstrated that plasma membrane proteins localize to numerous coexisting domains that partially overlap (Spira *et al.*, 2012). The lateral segregation of most proteins is strongly dependent on the lipid composition of the plasma membrane. In addition, the domain association of transmembrane proteins depends on their transmembrane sequence and is important for proper protein function.

The mechanism of Slm1 relocalization between two distinct plasma membrane compartments

Slm proteins leave eisosomes upon membrane stress or during inhibition of sphingolipid synthesis and activate TORC2 in the MCT. It is not clear, however, how Slm proteins perceive

plasma membrane stretch, or how they sense the altered lipid composition of the plasma membrane. How is the relocalization of Slm proteins mechanistically achieved?
It is unlikely that Slm proteins directly bind to complex sphingolipids, since Slm proteins are associated with the cytosolic leaflet of the plasma membrane, while complex sphingolipids predominately reside in the outer membrane leaflet. Proteins are able to indirectly perceive changes in the lipid composition of the plasma membrane. One example is the tetra-spanning membrane protein Nce102, which relocalizes from eisosomes to the remaining plasma membrane in response to sphingolipid synthesis inhibition (Frohlich *et al.*, 2009). In this case, decreased sphingolipid levels may reduce membrane thickness and/or abate the formation of sphingolipid-dependant microdomains, hence alter the localization of transmembrane proteins.

Since Slm proteins are peripheral membrane proteins, they rather sense structural changes of the plasma membrane. Evidence suggests that the furrow-like invaginations of eisosomes act as a sink for Slm proteins. As soon as eisosome formation occurs in the growing yeast bud, Slm proteins start to cluster there. In *pil1Δ* mutant cells, the eisosome pattern collapses and the remaining eisosome proteins form few remnants at huge plasma membrane invaginations (Walther *et al.*, 2006). These fewer, but larger invaginations recruit the same fraction of Slm proteins as normal eisosomes in wild type cells, since neither the concentration of Slm molecules in the MCT, nor TORC2 activity are altered in *pil1Δ* mutant cells (unpublished data of D. Berchtold and M. Piccolis).

How are Slm proteins recruited to eisosomal invaginations? So far, no physical interactions between Slm proteins and eisosome components were found. One interesting possibility though arises from a recent bioinformatics study revealing structural similarity between the SLM-domain of Slm proteins and bin-amphiphysin-rvs (BAR)-domains (Olivera-Couto *et al.*, 2011). BAR-domains bind to and/or induce membrane curvature. The putative BAR-domain of Slm proteins could potentially bind to the curved membrane at eisosomal furrows, thus recruiting Slm proteins to these domains. Consistent with this model, the SLM/BAR-domain is essential for Slm1 function and foci formation at eisosomes (unpublished data of D. Berchtold). If Slm proteins are recruited to eisosomal furrows by their putative BAR-domains, how are they released? One possibility is that membrane stretch flattens eisosomal invaginations, either through the disassembly of the protein coat or through physical forces. The decreased

curvature at eisosomes could reduce the affinity between the SLM/BAR-domain and eisosomal membranes and therefore trigger the relocalization of Slm proteins toward the MCT. Alternatively, a phosphorylation event could lead to a structural change in the SLM/BAR-domain. The altered conformation of the SLM/BAR-domain could then prevent Slm proteins from associating with curved membranes.

Together, the relocalization of Slm proteins between two mutually exclusive plasma membrane domains is an efficient mechanism to regulate TORC2 activity. Under normal conditions, the amount of Slm molecules in either domain is balanced to maintain low levels of TORC2 activity. Upon conditions that require a fast cellular response, for instance mechanical stretch or hypo-osmotic shock, Slm proteins are rapidly released from eisosomes to activate TORC2.

Eisosomes may represent functional analogs to mammalian caveolae

Eisosomes negatively affect signaling at the plasma membrane. The recruitment of Slm proteins to eisosomes prevents them from activating TORC2. Likewise, the recruitment of the transmembrane protein Nce102 to eisosomes inhibits Pkh kinase activity (Frohlich *et al.*, 2009). Upon pharmacological inhibition of sphingolipid synthesis, Nce102 is released from eisosomes, thereby indirectly activating Pkh kinases (Frohlich *et al.*, 2009).

The capacity of eisosomes to segregate and release proteins upon certain stimuli might be evolutionary conserved in mammalian cells. Caveolae likewise recruit signaling proteins, and the localization to these microdomains negatively affects their function. Endothelial nitric oxide synthase (eNOS), for instance, is targeted to caveolae by its lipid modifications (myristoylation and palmitoylation) and binds to the scaffolding domain of caveolin (Garcia-Cardena *et al.*, 1997). This interaction prevents eNOS activation by calmodulin, thus inhibits the production of the second messenger nitric oxide (NO). Consistently, eNOS shows higher activity in caveolin knock out cells (Sowa *et al.*, 2001).

Eisosomes and caveolae also share similarities at the structural level. Both microdomains are stable, protein-coated plasma membrane invaginations. The formation of both eisosomes and caveolae occurs through protein self-assembly of Pil1/Lsp1 and caveolin/cavin, respectively (Figure 8). Pil1/Lsp1 and caveolin are able to bind to lipids. Through their self-assembly at the plasma membrane, they might locally enrich certain lipids in eisosomes and caveolae,

respectively. In yeast, ergosterol was found to concentrate at eisosomes (Grossmann *et al.*, 2007). In addition, Pil1 and Lsp1 preferentially bind to phosphoinositide-containing membranes, thus may cluster PtdIns(4,5)P_2 at eisosomes (Karotki *et al.*, 2011). Caveolins directly bind to and enrich cholesterol at caveolae (Murata *et al.*, 1995; Ikonen and Parton, 2000). Since eisosomes and caveolae enrich and store certain lipids in plasma membrane invaginations, they might represent reservoirs to supply the cell with membrane lipids when needed.

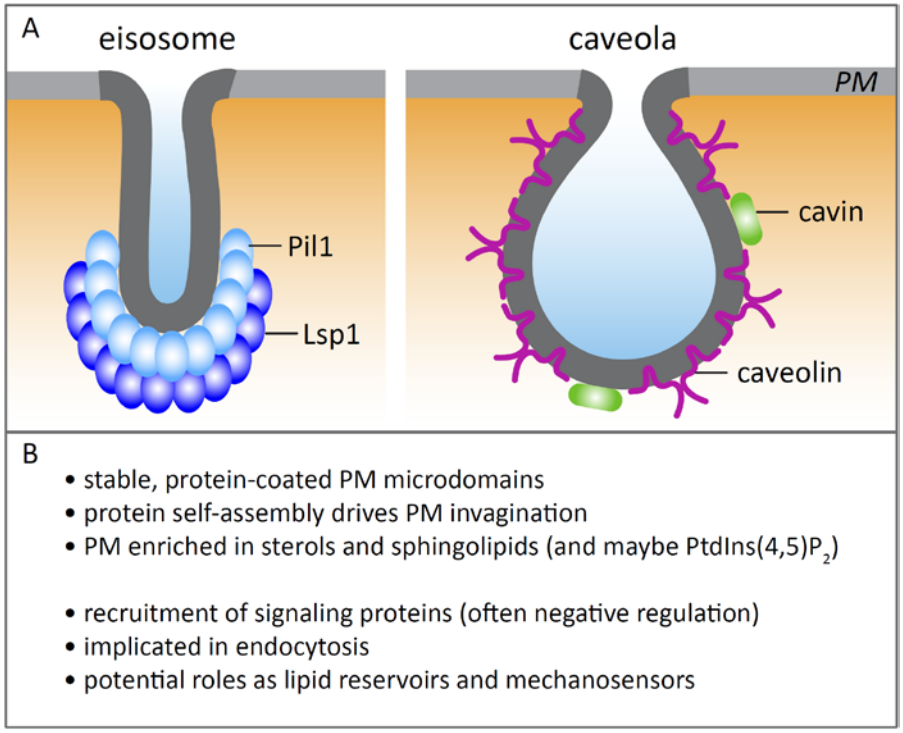

Figure 8: Comparison between eisosomes and caveolae. A) Cartoon illustrating the furrow-like invagination of an eisosome and a flask-shaped caveola at the plasma membrane (PM, grey). Proteins involved in eisosome or caveola formation are indicated. B) Common morphological features and proposed functions of eisosomes and caveolae are listed. PM: plasma membrane.

Caveolae are most abundant in adipocytes, where they play important roles in fatty acid uptake, transport and storage (Scherer *et al.*, 1994; Pilch *et al.*, 2011), but are also particularly abundant in cells exposed to mechanical stress, such as endothelial or muscle cells. These cells respond to chronic shear stress by increasing the number of caveolar invaginations at the plasma membrane (Boyd *et al.*, 2003; Rizzo *et al.*, 2003). Sinha *et al.* showed that acute mechanical stress or osmotic swelling leads to the disappearance of caveolae and the subsequent release of caveolins (Sinha *et al.*, 2011). The relief of stress leads to actin- and ATP-dependant

reassembly of caveolae. These findings suggest that the flask-like invaginations of caveolae represent membrane reservoirs and mechanosensors (Parton and Simons, 2007). The flattening of caveolar invaginations could instantaneously provide surface area upon acute plasma membrane stretch. This rapid response may protect endothelial and muscle cells from rupturing until slower processes, such as sphingolipid synthesis or exocytosis, are increased and counteract membrane stress. Since eisosomal furrows constitute a membrane reservoir similar to caveolar invaginations, eisosomes might as well act as mechanosensors. Fluorescence microscopy revealed that eisosomes partially disassemble during pharmacological inhibition of sphingolipid synthesis. At an ultrastructural level, however, it has not been investigated yet whether eisosomal furrows disappear or exhibit reduced curvature upon decreased sphingolipid levels or membrane stress.

Together, the common structural and functional attributes of eisosomes and caveolae represent intriguing parallels indicating that caveolae might represent functional analogs of eisosomes. Though only identified in fungi so far, the structure and function of eisosomes thus might be evolutionary conserved in mammalian cells.

The role of TORC2 in plasma membrane homeostasis

TORC2 is part of a sphingolipid-regulating feedback loop

Recent work by the Weisman lab elegantly demonstrated a feedback mechanism regulating the first and rate-limiting step in sphingolipid biosynthesis (Breslow *et al.*, 2010). They identified the conserved and essential Orm proteins as key regulators of the serine palmitoyl transferase (SPT) complex. Orm proteins interact with and inhibit SPT. In response to decreased sphingolipid levels, Orm1 and Orm2 are hyper-phosphorylated and release SPT, which results in an increased and compensating production of sphingolipids (Breslow *et al.*, 2010). Our data revealed that Orm1 hyper-phosphorylation upon myriocin treatment is largely mediated by Ypk1, and this finding is supported by another study (Roelants *et al.*, 2011). Together, these data place the Slm-mediated TORC2-Ypk1 activation upstream of Orm proteins, thus adding a sensing mechanism to the sphingolipid-regulating feedback loop (Figure 9).

Breslow and colleagues discovered another potential layer of regulation, which may coordinate phosphoinositide levels with sphingolipid synthesis (Breslow *et al.*, 2010). They discovered that SPT, together with the Orm proteins and the PtdIns(4)P phosphatase Sac1, forms a complex termed SPOTS, which is required for SPT inhibition. Though the regulation of Sac1 association with the SPOTS complex remains undefined, one could hypothesize that its release not only triggers activation of SPT but also impacts on phosphoinositide-dependant processes. Sac1 translocation from the ER to the Golgi apparatus, for instance, leads to locally reduced PtdIns(4)P levels and therefore decelerates the secretory pathway. Sac1 may also play a role in elevating the levels of PtdIns-derived phosphoinositol, which is required for the generation of IPC (Brice *et al.*, 2009).

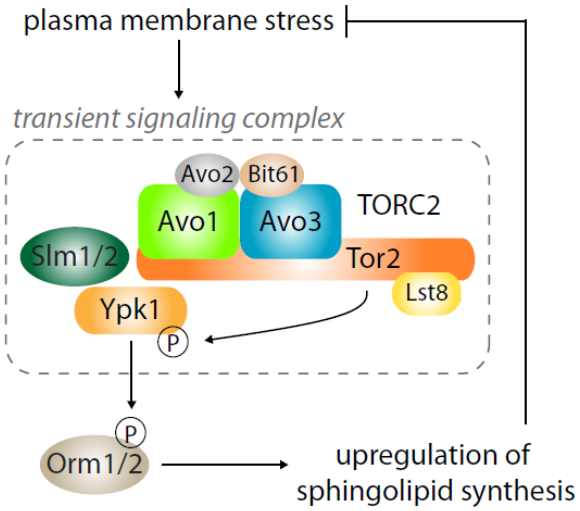

Figure 9: TORC2 is part of a sphingolipid-regulating feedback loop. Plasma membrane stress triggers the formation of a transient signaling complex consisting of Slm proteins, TORC2 and Ypk kinases. TORC2 activates Ypk kinases, which in turn activate Orm proteins, resulting in the upregulation of sphingolipid synthesis and the subsequent relieve of membrane stress.

Aronova and colleagues proposed a “feed-forward” regulated activation of ceramide synthase through a dual role of long chain bases (Aronova *et al.*, 2008). According to their model, accumulation of long chain bases triggers their conversion into ceramides by substrate-induced activation of ceramide synthase. Additionally, since PHS was demonstrated to stimulate the activity of AGC kinases, such as Pkh1/2 and Ypk1/2, *in vitro* (Liu *et al.*, 2005), long chain bases could indirectly - via Ypk kinases - account for increased ceramide synthase productivity. This

potential positive feedback mechanism integrates well as part of the negative, TORC2-controlled feedback regulation of sphingolipid levels (Figure 10).

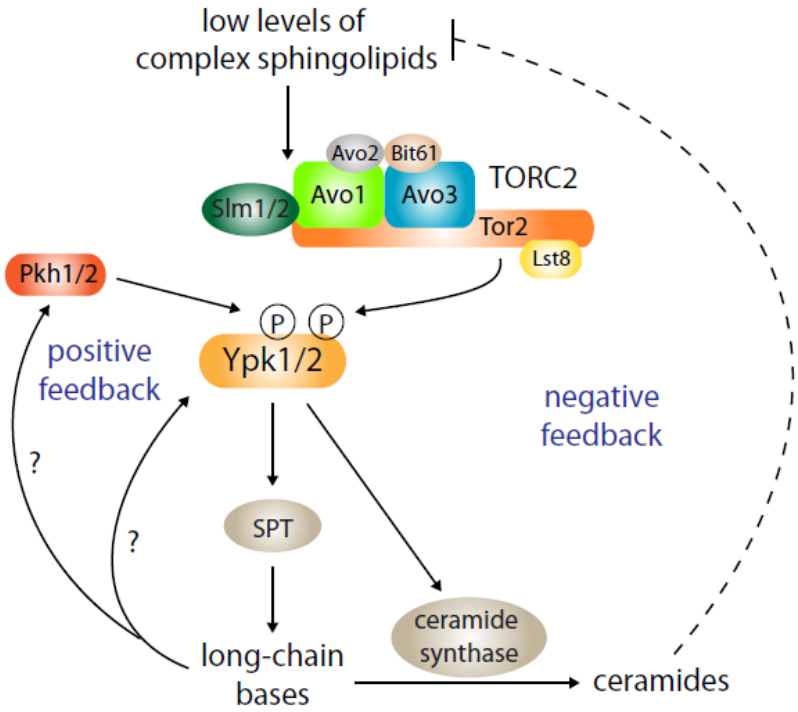

Figure 10: Positive and negative feedback loops may function in sphingolipid homeostasis. Decreased sphingolipid levels trigger the Slm-mediated activation of TORC2 and Ypk kinases. Ypk kinases activate the SPT complex via Orm protein phosphorylation, resulting in increased production of long chain bases, and in increased substrate-stimulated ceramide synthase activity. Long chain bases might work in a positive feedback mechanism by stimulating their own synthesis via the activation of Pkh1/2 and Ypk1/2. The resulting elevated levels of ceramides and complex sphingolipids negatively feed back and counteract TORC2 activation.

In this model, decreased sphingolipid levels lead to the activation of the TORC2-Ypk signaling pathway and to increased generation of long chain bases through activated SPT. Elevated levels of long chain bases may additionally contribute to Ypk kinase activation by stimulating Ypk autophosphorylation as well as the activity of their upstream kinases Pkh1/2. At the same time, Ypk kinases also activate the ceramide synthase complex, which converts long chain bases to ceramides. Thus, the positive feedback loop enhances ceramide production, ultimately leading to increased complex sphingolipid levels, which restore the plasma membrane composition and counteract TORC2 activation. However, since we found that TORC2 is not only activated upon inhibition of complex sphingolipid synthesis, but also upon membrane stretch, TORC2 rather

responds to plasma membrane availability. In this case, TORC2 locally perceives a lack of membrane and thereupon stimulates the biosynthesis of sphingolipids, which contribute to the generation of more membrane.

The substrate-feedback regulation appears to be a common principle in the regulation of TOR kinase. TORC1 acts in a negative feedback loop regulating ribosome biogenesis by activating two downstream targets, the transcriptional activator Sfp1 and Sch9 kinase (Lempiainen *et al.*, 2009). Both TORC1 effectors function in parallel to promote expression of genes coding for ribosomal and ribosome biogenesis proteins. Together, Sfp1 and Sch9 positively control translation efficiency in growing cells under favorable nutrient conditions. Loss of Sfp1-mediated transcriptional function and a concomitant decrease in ribosome biogenesis feeds back to TORC1-mediated hyper-phosphorylation of Sch9. In addition, a non-functional Sch9 mutant was found to be hyper-phosphorylated by TORC1 (Lempiainen *et al.*, 2009). This observation is similar to our data showing that a loss-of-function mutant of Ypk1 triggers its hyper-phosphorylation by TORC2. Hence, both TORC1 and TORC2 seem to constantly monitor the levels of basic cellular components (ribosomes and membranes/lipids, respectively) and adjust their synthesis rate according to cellular demand in a feedback mechanism.

In higher eukaryotes, there is no data so far supporting a direct role of TORC2 in the activation of ceramide synthase and/or plasma membrane homeostasis (Breslow and Weissman, 2010). However, the downstream target of mammalian TORC2 (mTORC2) and potential ortholog of yeast Ypk kinases, protein kinase B (PKB)/Akt, is an effector of the bioactive lipids ceramide and sphingosine 1-phosphate (S1P). Both metabolites derive from sphingosine, but have antagonizing functions in the regulation of PKB/Akt and hence cell growth (Bikman and Summers, 2011). S1P positively regulates cell metabolism and promotes nutrient (glucose) uptake via PKB/Akt activation. In contrast, various stress stimuli lead to a toxic accumulation of ceramides, which potently inhibit PKB/Akt and thus impair all PKB/Akt-mediated growth-stimulating functions, such as amino acid transport and protein synthesis. How the cells keep homeostasis between the two counteracting lipids S1P and ceramide, a concept referred to as sphingolipid rheostat, is still poorly understood (Fyrst and Saba, 2010). Therefore, it is interesting to test whether mTORC2 – via PKB/Akt – not only responds to but also regulates ceramide and S1P levels in a feedback loop similar to the one described for yeast (Figure 10).

TORC2 and Ypk kinases play conserved roles in mechanosensitive signaling

During cell division, growth-stimulating processes, such as protein translation and lipid synthesis, are upregulated to compensate the expanding cell's need for cellular components. TORC2 is a major spatial regulator of cell growth (Loewith and Hall, 2011). Though its upstream regulation is still poorly characterized, TORC2 is a key regulator of the actin cytoskeleton to establish cell polarity and control cell migration/chemotaxis in many organisms, such as *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster* and mammalian cells (Liu and Parent, 2011). The data presented here, together with other recent studies, suggest a novel role for TORC2 in the spatial regulation of growth apart from controlling the cytoskeleton.

We found TORC2 localizing to the plasma membrane where it responds to membrane stress and thereupon stimulates the generation of sphingolipids and membrane. This previously unrecognized role of TORC2 is consistent with its established function, the polarization of the actin cytoskeleton. Both processes require TORC2 activity specifically at sites of cell growth or membrane expansion, which in yeast mainly occurs at the bud tip during cell division. Since TORC2 foci are evenly distributed along the plasma membrane under various conditions tested, we assume that not all TORC2 signaling complexes are equally active, but are locally and temporally activated by interacting molecules, such as the Slm proteins. Interestingly, the same key players – Slm proteins and Ypk kinases – are required for both, actin cytoskeleton organization and sphingolipid/membrane homeostasis, suggesting that these two functions downstream of TORC2 may be connected or interdependent, for instance to facilitate the directed transport of newly generated membrane vesicles to sites of cell growth.

In mammalian cells, mTORC2 signals to PKB/Akt and serum glucocorticoid inducible kinase 1 (SGK1), both functional orthologs of yeast Ypk kinases (Jacinto and Lorberg, 2008). Both PKB/Akt and SGK1 play roles in mechanosensitive signaling. Mechanical stretch of epithelial cells, for instance, induces cluster formation and trans-activation of epidermal growth factor receptor (EGFR), followed by a rapid increase in PKB/Akt phosphorylation at both PDK1 and PDK2 sites (Kippenberger *et al.*, 2005). In venous smooth muscle cells, SGK1 is activated by mTORC2 upon cyclic mechanical stretch (Cheng *et al.*, 2010).

Caveolae play an important role in the stretch-induced activation of kinases: Chronic shear stress in endothelial cells stimulates increased formation of caveolae, and phosphorylation of PKB/Akt in response to mechanical membrane stretch is faster in such preconditioned cells (Boyd *et al.*, 2003). Caveolin-1 is necessary for the formation of PKB/Akt-activating signaling complexes at focal adhesion sites during mechanical stretch of vascular smooth muscle cells (Sedding *et al.*, 2005). Consistently, down regulation of caveolin-1 diminishes PKB/Akt phosphorylation in response to membrane stress. In addition, membrane stretch induces caveolae- and EGFR-dependant PKB/Akt phosphorylation by mTORC2 (Zhang *et al.*, 2007). It is not yet clear, however, whether caveolae play a direct or indirect role in the activation of PKB/Akt. When ceramide is added to the cells, PKB/Akt is recruited to caveolae and remains inactive there (Hajduch *et al.*, 2008). Thus, caveolae might act as repressive microdomains, which facilitate the spatial separation of PKB/Akt from its upstream kinases.

Together, it is likely that the mechanism of TORC2/Ypk1 activation upon plasma membrane stress is evolutionary conserved in mammalian cells. Our findings highlight the functional parallels between eisosomes and caveolae not only as signaling platforms, but also as mechanosensitive membrane reservoirs. In yeast, Slm proteins are required for the perception of plasma membrane stress and the subsequent activation of TORC2 and Ypk1. Sequence homology searches, however, did not reveal a functional ortholog of Slm proteins in mammalian cells. Hence, proteins required for mTORC2 activation remain to be identified. It will be exciting to investigate the mechanism of mTORC2 activation upon membrane stress and to determine whether mTORC2 also maintains plasma membrane homeostasis in mammalian cells.

References

Aronova, S., K. Wedaman, S. Anderson, J. Yates, 3rd, and T. Powers. 2007. Probing the membrane environment of the TOR kinases reveals functional interactions between TORC1, actin, and membrane trafficking in Saccharomyces cerevisiae. *Mol Biol Cell*. 18:2779-2794.

Aronova, S., K. Wedaman, P.A. Aronov, K. Fontes, K. Ramos, B.D. Hammock, and T. Powers. 2008. Regulation of ceramide biosynthesis by TOR complex 2. *Cell Metab*. 7:148-158.

Audhya, A., R. Loewith, A.B. Parsons, L. Gao, M. Tabuchi, H. Zhou, C. Boone, M.N. Hall, and S.D. Emr. 2004. Genome-wide lethality screen identifies new PI4,5P2 effectors that regulate the actin cytoskeleton. *EMBO J*. 23:3747-3757.

Bagnat, M., A. Chang, and K. Simons. 2001. Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast. *Mol Biol Cell*. 12:4129-4138.

Balda, M.S., J.A. Whitney, C. Flores, S. Gonzalez, M. Cereijido, and K. Matter. 1996. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol*. 134:1031-1049.

Barral, Y., V. Mermall, M.S. Mooseker, and M. Snyder. 2000. Compartmentalization of the cell cortex by septins is required for maintenance of cell polarity in yeast. *Mol Cell*. 5:841-851.

Bastiani, M., and R.G. Parton. 2010. Caveolae at a glance. *J Cell Sci*. 123:3831-3836.

Beeler, T., D. Bacikova, K. Gable, L. Hopkins, C. Johnson, H. Slife, and T. Dunn. 1998. The Saccharomyces cerevisiae TSC10/YBR265w gene encoding 3-ketosphinganine reductase is identified in a screen for temperature-sensitive suppressors of the Ca2+-sensitive csg2Delta mutant. *J Biol Chem*. 273:30688-30694.

Bikman, B.T., and S.A. Summers. 2011. Ceramides as modulators of cellular and whole-body metabolism. *J Clin Invest*. 121:4222-4230.

Binda, M., M.P. Peli-Gulli, G. Bonfils, N. Panchaud, J. Urban, T.W. Sturgill, R. Loewith, and C. De Virgilio. 2009. The Vam6 GEF controls TORC1 by activating the EGO complex. *Mol Cell*. 35:563-573.

Boyd, N.L., H. Park, H. Yi, Y.C. Boo, G.P. Sorescu, M. Sykes, and H. Jo. 2003. Chronic shear induces caveolae formation and alters ERK and Akt responses in endothelial cells. *Am J Physiol Heart Circ Physiol*. 285:H1113-1122.

Brach, T., T. Specht, and M. Kaksonen. 2011. Reassessment of the role of plasma membrane domains in the regulation of vesicular traffic in yeast. *J Cell Sci*. 124:328-337.

Breslow, D.K., S.R. Collins, B. Bodenmiller, R. Aebersold, K. Simons, A. Shevchenko, C.S. Ejsing, and J.S. Weissman. 2010. Orm family proteins mediate sphingolipid homeostasis. *Nature*. 463:1048-1053.

Breslow, D.K., and J.S. Weissman. 2010. Membranes in balance: mechanisms of sphingolipid homeostasis. *Mol Cell*. 40:267-279.

Brice, S.E., C.W. Alford, and L.A. Cowart. 2009. Modulation of sphingolipid metabolism by the phosphatidylinositol-4-phosphate phosphatase Sac1p through regulation of phosphatidylinositol in Saccharomyces cerevisiae. *J Biol Chem*. 284:7588-7596.

Brown, D.A., and J.K. Rose. 1992. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell*. 68:533-544.

Bultynck, G., V.L. Heath, A.P. Majeed, J.M. Galan, R. Haguenauer-Tsapis, and M.S. Cyert. 2006. Slm1 and slm2 are novel substrates of the calcineurin phosphatase required for heat stress-induced endocytosis of the yeast uracil permease. *Mol Cell Biol*. 26:4729-4745.

Casamayor, A., P.D. Torrance, T. Kobayashi, J. Thorner, and D.R. Alessi. 1999. Functional counterparts of mammalian protein kinases PDK1 and SGK in budding yeast. *Curr Biol*. 9:186-197.

Cheng, J., Y. Wang, Y. Ma, B.T. Chan, M. Yang, A. Liang, L. Zhang, H. Li, and J. Du. 2010. The mechanical stress-activated serum-, glucocorticoid-regulated kinase 1 contributes to neointima formation in vein grafts. *Circ Res*. 107:1265-1274.

Couet, J., S. Li, T. Okamoto, T. Ikezu, and M.P. Lisanti. 1997. Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins. *J Biol Chem*. 272:6525-6533.

Cowart, L.A., and L.M. Obeid. 2007. Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. *Biochim Biophys Acta*. 1771:421-431.

Cowart, L.A., M. Shotwell, M.L. Worley, A.J. Richards, D.J. Montefusco, Y.A. Hannun, and X. Lu. 2010. Revealing a signaling role of phytosphingosine-1-phosphate in yeast. *Mol Syst Biol*. 6:349.

Daquinag, A., M. Fadri, S.Y. Jung, J. Qin, and J. Kunz. 2007. The yeast PH domain proteins Slm1 and Slm2 are targets of sphingolipid signaling during the response to heat stress. *Mol Cell Biol*. 27:633-650.

De Virgilio, C., and R. Loewith. 2006. Cell growth control: little eukaryotes make big contributions. *Oncogene*. 25:6392-6415.

deHart, A.K., J.D. Schnell, D.A. Allen, and L. Hicke. 2002. The conserved Pkh-Ypk kinase cascade is required for endocytosis in yeast. *J Cell Biol*. 156:241-248.

deHart, A.K., J.D. Schnell, D.A. Allen, J.Y. Tsai, and L. Hicke. 2003. Receptor internalization in yeast requires the Tor2-Rho1 signaling pathway. *Mol Biol Cell*. 14:4676-4684.

Dickson, R.C., E.E. Nagiec, M. Skrzypek, P. Tillman, G.B. Wells, and R.L. Lester. 1997a. Sphingolipids are potential heat stress signals in Saccharomyces. *J Biol Chem*. 272:30196-30200.

Dickson, R.C., E.E. Nagiec, G.B. Wells, M.M. Nagiec, and R.L. Lester. 1997b. Synthesis of mannose-(inositol-P)2-ceramide, the major sphingolipid in Saccharomyces cerevisiae, requires the IPT1 (YDR072c) gene. *J Biol Chem*. 272:29620-29625.

Dickson, R.C., C. Sumanasekera, and R.L. Lester. 2006. Functions and metabolism of sphingolipids in Saccharomyces cerevisiae. *Prog Lipid Res*. 45:447-465.

Douglass, A.D., and R.D. Vale. 2005. Single-molecule microscopy reveals plasma membrane microdomains created by protein-protein networks that exclude or trap signaling molecules in T cells. *Cell*. 121:937-950.

Eggeling, C., C. Ringemann, R. Medda, G. Schwarzmann, K. Sandhoff, S. Polyakova, V.N. Belov, B. Hein, C. von Middendorff, A. Schonle, and S.W. Hell. 2009. Direct observation of the nanoscale dynamics of membrane lipids in a living cell. *Nature*. 457:1159-1162.

Ejsing, C.S., J.L. Sampaio, V. Surendranath, E. Duchoslav, K. Ekroos, R.W. Klemm, K. Simons, and A. Shevchenko. 2009. Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry. *Proc Natl Acad Sci U S A*. 106:2136-2141.

Fadri, M., A. Daquinag, S. Wang, T. Xue, and J. Kunz. 2005. The pleckstrin homology domain proteins Slm1 and Slm2 are required for actin cytoskeleton organization in yeast and bind phosphatidylinositol-4,5-bisphosphate and TORC2. *Mol Biol Cell*. 16:1883-1900.

Ferrell, J.E., Jr., and W.H. Huestis. 1984. Phosphoinositide metabolism and the morphology of human erythrocytes. *J Cell Biol*. 98:1992-1998.

Foster, L.J., C.L. De Hoog, and M. Mann. 2003. Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc Natl Acad Sci U S A*. 100:5813-5818.

Friant, S., R. Lombardi, T. Schmelzle, M.N. Hall, and H. Riezman. 2001. Sphingoid base signaling via Pkh kinases is required for endocytosis in yeast. *EMBO J*. 20:6783-6792.

Frohlich, F., K. Moreira, P.S. Aguilar, N.C. Hubner, M. Mann, P. Walter, and T.C. Walther. 2009. A genome-wide screen for genes affecting eisosomes reveals Nce102 function in sphingolipid signaling. *J Cell Biol*. 185:1227-1242.

Frost, A., V.M. Unger, and P. De Camilli. 2009. The BAR domain superfamily: membrane-molding macromolecules. *Cell*. 137:191-196.

Fujita, T., K. Inoue, S. Yamamoto, T. Ikumoto, S. Sasaki, R. Toyama, K. Chiba, Y. Hoshino, and T. Okumoto. 1994. Fungal metabolites. Part 11. A potent immunosuppressive activity found in Isaria sinclairii metabolite. *J Antibiot (Tokyo)*. 47:208-215.

Fujiwara, T., K. Ritchie, H. Murakoshi, K. Jacobson, and A. Kusumi. 2002. Phospholipids undergo hop diffusion in compartmentalized cell membrane. *J Cell Biol*. 157:1071-1081.

Funato, K., and H. Riezman. 2001. Vesicular and nonvesicular transport of ceramide from ER to the Golgi apparatus in yeast. *J Cell Biol*. 155:949-959.

Futerman, A.H., and Y.A. Hannun. 2004. The complex life of simple sphingolipids. *EMBO Rep*. 5:777-782.

Fyrst, H., and J.D. Saba. 2010. An update on sphingosine-1-phosphate and other sphingolipid mediators. *Nat Chem Biol*. 6:489-497.

Gaigg, B., B. Timischl, L. Corbino, and R. Schneiter. 2005. Synthesis of sphingolipids with very long chain fatty acids but not ergosterol is required for routing of newly synthesized plasma membrane ATPase to the cell surface of yeast. *J Biol Chem*. 280:22515-22522.

Gaigg, B., A. Toulmay, and R. Schneiter. 2006. Very long-chain fatty acid-containing lipids rather than sphingolipids per se are required for raft association and stable surface transport of newly synthesized plasma membrane ATPase in yeast. *J Biol Chem*. 281:34135-34145.

Gandhavadi, M., D. Allende, A. Vidal, S.A. Simon, and T.J. McIntosh. 2002. Structure, composition, and peptide binding properties of detergent soluble bilayers and detergent resistant rafts. *Biophys J*. 82:1469-1482.

Garcia-Cardena, G., P. Martasek, B.S. Masters, P.M. Skidd, J. Couet, S. Li, M.P. Lisanti, and W.C. Sessa. 1997. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J Biol Chem*. 272:25437-25440.

Grossmann, G., M. Opekarova, J. Malinsky, I. Weig-Meckl, and W. Tanner. 2007. Membrane potential governs lateral segregation of plasma membrane proteins and lipids in yeast. *EMBO J*. 26:1-8.

Guan, X.L., and M.R. Wenk. 2006. Mass spectrometry-based profiling of phospholipids and sphingolipids in extracts from Saccharomyces cerevisiae. *Yeast*. 23:465-477.

Gupta, N., and A.L. DeFranco. 2003. Visualizing lipid raft dynamics and early signaling events during antigen receptor-mediated B-lymphocyte activation. *Mol Biol Cell*. 14:432-444.

Hajduch, E., S. Turban, X. Le Liepvre, S. Le Lay, C. Lipina, N. Dimopoulos, I. Dugail, and H.S. Hundal. 2008. Targeting of PKCzeta and PKB to caveolin-enriched microdomains represents a crucial step underpinning the disruption in PKB-directed signalling by ceramide. *Biochem J*. 410:369-379.

Hanada, K. 2003. Serine palmitoyltransferase, a key enzyme of sphingolipid metabolism. *Biochim Biophys Acta*. 1632:16-30.

Hanada, K., K. Kumagai, N. Tomishige, and T. Yamaji. 2009. CERT-mediated trafficking of ceramide. *Biochim Biophys Acta*. 1791:684-691.

Harvey, R.D., and S.C. Calaghan. 2012. Caveolae create local signalling domains through their distinct protein content, lipid profile and morphology. *J Mol Cell Cardiol*. 52:366-375.

Hill, M.M., M. Bastiani, R. Luetterforst, M. Kirkham, A. Kirkham, S.J. Nixon, P. Walser, D. Abankwa, V.M. Oorschot, S. Martin, J.F. Hancock, and R.G. Parton. 2008. PTRF-Cavin, a conserved cytoplasmic protein required for caveola formation and function. *Cell*. 132:113-124.

Ikonen, E., and R.G. Parton. 2000. Caveolins and cellular cholesterol balance. *Traffic*. 1:212-217.

Jacinto, E., R. Loewith, A. Schmidt, S. Lin, M.A. Ruegg, A. Hall, and M.N. Hall. 2004. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat Cell Biol*. 6:1122-1128.

Jacinto, E., and A. Lorberg. 2008. TOR regulation of AGC kinases in yeast and mammals. *Biochem J*. 410:19-37.

Jacobson, K., O.G. Mouritsen, and R.G. Anderson. 2007. Lipid rafts: at a crossroad between cell biology and physics. *Nat Cell Biol*. 9:7-14.

Jorgensen, P., I. Rupes, J.R. Sharom, L. Schneper, J.R. Broach, and M. Tyers. 2004. A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes Dev*. 18:2491-2505.

Kaksonen, M., C.P. Toret, and D.G. Drubin. 2005. A modular design for the clathrin- and actin-mediated endocytosis machinery. *Cell*. 123:305-320.

Kaksonen, M., C.P. Toret, and D.G. Drubin. 2006. Harnessing actin dynamics for clathrin-mediated endocytosis. *Nat Rev Mol Cell Biol*. 7:404-414.

Kamada, Y., Y. Fujioka, N.N. Suzuki, F. Inagaki, S. Wullschleger, R. Loewith, M.N. Hall, and Y. Ohsumi. 2005. Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol Cell Biol*. 25:7239-7248.

Karotki, L., J.T. Huiskonen, C.J. Stefan, N.E. Ziolkowska, R. Roth, M.A. Surma, N.J. Krogan, S.D. Emr, J. Heuser, K. Grunewald, and T.C. Walther. 2011. Eisosome proteins assemble into a membrane scaffold. *J Cell Biol*. 195:889-902.

Kippenberger, S., S. Loitsch, M. Guschel, J. Muller, Y. Knies, R. Kaufmann, and A. Bernd. 2005. Mechanical stretch stimulates protein kinase B/Akt phosphorylation in epidermal cells via angiotensin II type 1 receptor and epidermal growth factor receptor. *J Biol Chem*. 280:3060-3067.

Kornberg, R.D., and H.M. McConnell. 1971. Lateral diffusion of phospholipids in a vesicle membrane. *Proc Natl Acad Sci U S A*. 68:2564-2568.

Kunz, J., U. Schneider, I. Howald, A. Schmidt, and M.N. Hall. 2000. HEAT repeats mediate plasma membrane localization of Tor2p in yeast. *J Biol Chem*. 275:37011-37020.

Kusumi, A., Y. Sako, and M. Yamamoto. 1993. Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. *Biophys J*. 65:2021-2040.

Kutateladze, T.G. 2010. Translation of the phosphoinositide code by PI effectors. *Nat Chem Biol*. 6:507-513.

Laude, A.J., and I.A. Prior. 2004. Plasma membrane microdomains: organization, function and trafficking. *Mol Membr Biol*. 21:193-205.

Lee, M.C., S. Hamamoto, and R. Schekman. 2002. Ceramide biosynthesis is required for the formation of the oligomeric H+-ATPase Pma1p in the yeast endoplasmic reticulum. *J Biol Chem*. 277:22395-22401.

Lempiainen, H., A. Uotila, J. Urban, I. Dohnal, G. Ammerer, R. Loewith, and D. Shore. 2009. Sfp1 interaction with TORC1 and Mrs6 reveals feedback regulation on TOR signaling. *Mol Cell*. 33:704-716.

Lingwood, D., and K. Simons. 2010. Lipid rafts as a membrane-organizing principle. *Science*. 327:46-50.

Liu, K., X. Zhang, R.L. Lester, and R.C. Dickson. 2005. The sphingoid long chain base phytosphingosine activates AGC-type protein kinases in Saccharomyces cerevisiae including Ypk1, Ypk2, and Sch9. *J Biol Chem*. 280:22679-22687.

Liu, L., D. Brown, M. McKee, N.K. Lebrasseur, D. Yang, K.H. Albrecht, K. Ravid, and P.F. Pilch. 2008. Deletion of Cavin/PTRF causes global loss of caveolae, dyslipidemia, and glucose intolerance. *Cell Metab*. 8:310-317.

Liu, L., and C.A. Parent. 2011. Review series: TOR kinase complexes and cell migration. *J Cell Biol*. 194:815-824.

Liu, L., and P.F. Pilch. 2008. A critical role of cavin (polymerase I and transcript release factor) in caveolae formation and organization. *J Biol Chem*. 283:4314-4322.

Loewith, R., and M.N. Hall. 2011. Target of rapamycin (TOR) in nutrient signaling and growth control. *Genetics*. 189:1177-1201.

Loewith, R., E. Jacinto, S. Wullschleger, A. Lorberg, J.L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, and M.N. Hall. 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol Cell*. 10:457-468.

Malinska, K., J. Malinsky, M. Opekarova, and W. Tanner. 2003. Visualization of protein compartmentation within the plasma membrane of living yeast cells. *Mol Biol Cell*. 14:4427-4436.

Malinska, K., J. Malinsky, M. Opekarova, and W. Tanner. 2004. Distribution of Can1p into stable domains reflects lateral protein segregation within the plasma membrane of living S. cerevisiae cells. *J Cell Sci*. 117:6031-6041.

Mansy, S.S. 2010. Membrane transport in primitive cells. *Cold Spring Harb Perspect Biol*. 2:a002188.

Martin, D.E., and M.N. Hall. 2005. The expanding TOR signaling network. *Curr Opin Cell Biol*. 17:158-166.

Meier, K.D., O. Deloche, K. Kajiwara, K. Funato, and H. Riezman. 2006. Sphingoid base is required for translation initiation during heat stress in Saccharomyces cerevisiae. *Mol Biol Cell*. 17:1164-1175.

Meshulam, T., M.R. Breen, L. Liu, R.G. Parton, and P.F. Pilch. 2011. Caveolins/caveolae protect adipocytes from fatty acid-mediated lipotoxicity. *J Lipid Res*. 52:1526-1532.

Meshulam, T., J.R. Simard, J. Wharton, J.A. Hamilton, and P.F. Pilch. 2006. Role of caveolin-1 and cholesterol in transmembrane fatty acid movement. *Biochemistry*. 45:2882-2893.

Monier, S., D.J. Dietzen, W.R. Hastings, D.M. Lublin, and T.V. Kurzchalia. 1996. Oligomerization of VIP21-caveolin in vitro is stabilized by long chain fatty acylation or cholesterol. *FEBS Lett*. 388:143-149.

Moreira, K.E., T.C. Walther, P.S. Aguilar, and P. Walter. 2009. Pil1 controls eisosome biogenesis. *Mol Biol Cell*. 20:809-818.

Mulet, J.M., D.E. Martin, R. Loewith, and M.N. Hall. 2006. Mutual antagonism of target of rapamycin and calcineurin signaling. *J Biol Chem*. 281:33000-33007.

Mulholland, J., D. Preuss, A. Moon, A. Wong, D. Drubin, and D. Botstein. 1994. Ultrastructure of the yeast actin cytoskeleton and its association with the plasma membrane. *J Cell Biol*. 125:381-391.

Munro, S. 2003. Lipid rafts: elusive or illusive? *Cell*. 115:377-388.

Murata, M., J. Peranen, R. Schreiner, F. Wieland, T.V. Kurzchalia, and K. Simons. 1995. VIP21/caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci U S A*. 92:10339-10343.

Nagiec, M.M., E.E. Nagiec, J.A. Baltisberger, G.B. Wells, R.L. Lester, and R.C. Dickson. 1997. Sphingolipid synthesis as a target for antifungal drugs. Complementation of the inositol phosphorylceramide synthase defect in a mutant strain of Saccharomyces cerevisiae by the AUR1 gene. *J Biol Chem*. 272:9809-9817.

Nagiec, M.M., M. Skrzypek, E.E. Nagiec, R.L. Lester, and R.C. Dickson. 1998. The LCB4 (YOR171c) and LCB5 (YLR260w) genes of Saccharomyces encode sphingoid long chain base kinases. *J Biol Chem*. 273:19437-19442.

Olivera-Couto, A., M. Grana, L. Harispe, and P.S. Aguilar. 2011. The eisosome core is composed of BAR domain proteins. *Mol Biol Cell*. 22:2360-2372.

Parton, R.G., and K. Simons. 2007. The multiple faces of caveolae. *Nat Rev Mol Cell Biol*. 8:185-194.

Pelkmans, L., and M. Zerial. 2005. Kinase-regulated quantal assemblies and kiss-and-run recycling of caveolae. *Nature*. 436:128-133.

Pilch, P.F., T. Meshulam, S. Ding, and L. Liu. 2011. Caveolae and lipid trafficking in adipocytes. *Clin Lipidol*. 6:49-58.

Rizzo, V., C. Morton, N. DePaola, J.E. Schnitzer, and P.F. Davies. 2003. Recruitment of endothelial caveolae into mechanotransduction pathways by flow conditioning in vitro. *Am J Physiol Heart Circ Physiol*. 285:H1720-1729.

Rodriguez-Boulan, E., and A. Musch. 2005. Protein sorting in the Golgi complex: shifting paradigms. *Biochim Biophys Acta*. 1744:455-464.

Rodriguez-Boulan, E., and W.J. Nelson. 1989. Morphogenesis of the polarized epithelial cell phenotype. *Science*. 245:718-725.

Roelants, F.M., D.K. Breslow, A. Muir, J.S. Weissman, and J. Thorner. 2011. Protein kinase Ypk1 phosphorylates regulatory proteins Orm1 and Orm2 to control sphingolipid homeostasis in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A*. 108:19222-19227.

Roelants, F.M., P.D. Torrance, N. Bezman, and J. Thorner. 2002. Pkh1 and Pkh2 differentially phosphorylate and activate Ypk1 and Ykr2 and define protein kinase modules required for maintenance of cell wall integrity. *Mol Biol Cell*. 13:3005-3028.

Sako, Y., and A. Kusumi. 1994. Compartmentalized structure of the plasma membrane for receptor movements as revealed by a nanometer-level motion analysis. *J Cell Biol*. 125:1251-1264.

Sarbassov, D.D., S.M. Ali, D.H. Kim, D.A. Guertin, R.R. Latek, H. Erdjument-Bromage, P. Tempst, and D.M. Sabatini. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr Biol*. 14:1296-1302.

Scherer, P.E., M.P. Lisanti, G. Baldini, M. Sargiacomo, C.C. Mastick, and H.F. Lodish. 1994. Induction of caveolin during adipogenesis and association of GLUT4 with caveolin-rich vesicles. *J Cell Biol*. 127:1233-1243.

Schuck, S., and K. Simons. 2004. Polarized sorting in epithelial cells: raft clustering and the biogenesis of the apical membrane. *J Cell Sci*. 117:5955-5964.

Sedding, D.G., J. Hermsen, U. Seay, O. Eickelberg, W. Kummer, C. Schwencke, R.H. Strasser, H. Tillmanns, and R.C. Braun-Dullaeus. 2005. Caveolin-1 facilitates mechanosensitive protein kinase B (Akt) signaling in vitro and in vivo. *Circ Res*. 96:635-642.

Shevchenko, A., and K. Simons. 2010. Lipidomics: coming to grips with lipid diversity. *Nat Rev Mol Cell Biol*. 11:593-598.

Simons, K., and G. van Meer. 1988. Lipid sorting in epithelial cells. *Biochemistry*. 27:6197-6202.

Singer, S.J., and G.L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science*. 175:720-731.

Sinha, B., D. Koster, R. Ruez, P. Gonnord, M. Bastiani, D. Abankwa, R.V. Stan, G. Butler-Browne, B. Vedie, L. Johannes, N. Morone, R.G. Parton, G. Raposo, P. Sens, C. Lamaze, and P. Nassoy. 2011. Cells respond to mechanical stress by rapid disassembly of caveolae. *Cell*. 144:402-413.

Sowa, G., M. Pypaert, and W.C. Sessa. 2001. Distinction between signaling mechanisms in lipid rafts vs. caveolae. *Proc Natl Acad Sci U S A*. 98:14072-14077.

Spira, F., N.S. Mueller, G. Beck, P. von Olshausen, J. Beig, and R. Wedlich-Soldner. 2012. Patchwork organization of the yeast plasma membrane into numerous coexisting domains. *Nat Cell Biol*. doi: 10.1038/ncb2487. [Epub ahead of print]

Stradalova, V., W. Stahlschmidt, G. Grossmann, M. Blazikova, R. Rachel, W. Tanner, and J. Malinsky. 2009. Furrow-like invaginations of the yeast plasma membrane correspond to membrane compartment of Can1. *J Cell Sci*. 122:2887-2894.

Sun, Y., R. Taniguchi, D. Tanoue, T. Yamaji, H. Takematsu, K. Mori, T. Fujita, T. Kawasaki, and Y. Kozutsumi. 2000. Sli2 (Ypk1), a homologue of mammalian protein kinase SGK, is a downstream kinase in the sphingolipid-mediated signaling pathway of yeast. *Mol Cell Biol*. 20:4411-4419.

Tabuchi, M., A. Audhya, A.B. Parsons, C. Boone, and S.D. Emr. 2006. The phosphatidylinositol 4,5-biphosphate and TORC2 binding proteins Slm1 and Slm2 function in sphingolipid regulation. *Mol Cell Biol*. 26:5861-5875.

Takizawa, P.A., J.L. DeRisi, J.E. Wilhelm, and R.D. Vale. 2000. Plasma membrane compartmentalization in yeast by messenger RNA transport and a septin diffusion barrier. *Science*. 290:341-344.

Tanoue, D., T. Kobayashi, Y. Sun, T. Fujita, H. Takematsu, and Y. Kozutsumi. 2005. The requirement for the hydrophobic motif phosphorylation of Ypk1 in yeast differs depending on the downstream events, including endocytosis, cell growth, and resistance to a sphingolipid biosynthesis inhibitor, ISP-1. *Arch Biochem Biophys*. 437:29-41.

Toret, C.P., and D.G. Drubin. 2006. The budding yeast endocytic pathway. *J Cell Sci*. 119:4585-4587.

Uemura, S., A. Kihara, J. Inokuchi, and Y. Igarashi. 2003. Csg1p and newly identified Csh1p function in mannosylinositol phosphorylceramide synthesis by interacting with Csg2p. *J Biol Chem*. 278:45049-45055.

Urban, J., A. Soulard, A. Huber, S. Lippman, D. Mukhopadhyay, O. Deloche, V. Wanke, D. Anrather, G. Ammerer, H. Riezman, J.R. Broach, C. De Virgilio, M.N. Hall, and R. Loewith. 2007. Sch9 is a major target of TORC1 in Saccharomyces cerevisiae. *Mol Cell*. 26:663-674.

van Meer, G., D.R. Voelker, and G.W. Feigenson. 2008. Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol*. 9:112-124.

Walther, T.C., J.H. Brickner, P.S. Aguilar, S. Bernales, C. Pantoja, and P. Walter. 2006. Eisosomes mark static sites of endocytosis. *Nature*. 439:998-1003.

Wang, D.S., R. Shaw, M. Hattori, H. Arai, K. Inoue, and G. Shaw. 1995. Binding of pleckstrin homology domains to WD40/beta-transducin repeat containing segments of the protein product of the Lis-1 gene. *Biochem Biophys Res Commun*. 209:622-629.

Wedaman, K.P., A. Reinke, S. Anderson, J. Yates, 3rd, J.M. McCaffery, and T. Powers. 2003. Tor kinases are in distinct membrane-associated protein complexes in Saccharomyces cerevisiae. *Mol Biol Cell*. 14:1204-1220.

Wullschleger, S., R. Loewith, W. Oppliger, and M.N. Hall. 2005. Molecular organization of target of rapamycin complex 2. *J Biol Chem*. 280:30697-30704.

Yu, J.W., J.M. Mendrola, A. Audhya, S. Singh, D. Keleti, D.B. DeWald, D. Murray, S.D. Emr, and M.A. Lemmon. 2004. Genome-wide analysis of membrane targeting by S. cerevisiae pleckstrin homology domains. *Mol Cell*. 13:677-688.

Zacharias, D.A., J.D. Violin, A.C. Newton, and R.Y. Tsien. 2002. Partitioning of lipid-modified monomeric GFPs into membrane microdomains of live cells. *Science*. 296:913-916.

Zanolari, B., S. Friant, K. Funato, C. Sutterlin, B.J. Stevenson, and H. Riezman. 2000. Sphingoid base synthesis requirement for endocytosis in Saccharomyces cerevisiae. *EMBO J*. 19:2824-2833.

Zhang, B., F. Peng, D. Wu, A.J. Ingram, B. Gao, and J.C. Krepinsky. 2007. Caveolin-1 phosphorylation is required for stretch-induced EGFR and Akt activation in mesangial cells. *Cell Signal*. 19:1690-1700.

Ziolkowska, N.E., L. Karotki, M. Rehman, J.T. Huiskonen, and T.C. Walther. 2011. Eisosome-driven plasma membrane organization is mediated by BAR domains. *Nat Struct Mol Biol*. 18:854-856.

Abbreviations

AGC family	protein kinases A, G, and C family
BAR-domain	bin-amphiphysin-rvs domain
DHS	dihydrosphingosine
EGFR	epidermal growth factor receptor
eNOS	endothelial nitric oxide synthase
ER	endoplasmatic reticulum
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
IPC	inositolphosphoceramide
$M(IP)_2C$	mannose-di-inositolphosphoceramide
MCC	membrane compartment occupied by Can1
MCP	membrane compartment occupied by Pma1
MCT	membrane compartment occupied by TOR complex 2
MIPC	mannose-inositolphosphoceramide
mTORC2	mammalian target of rapamycin kinase complex 2
PDK1	phosphoinositide-dependant kinase 1
PH-domain	pleckstrin homology domain
PHS	phytosphingosine
PKB	protein kinase B
PtdIns	phosphatidylinositol
PtdIns(4)P	phosphatidylinositol 4-phosphate
$PtdIns(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
S1P	sphingosine 1-phosphate
SGK1	serum glucocorticoid inducible kinase 1
SPOTS complex	complex consisting of serine palmitoyltransferase, Orm1/2, Tsc3, Sac1
SPT	serine palmitoyltransferase
TOR	target of rapamycin
TORC1/2	target of rapamycin kinase complex 1/2

Declaration of individual contribution

Publication I

Berchtold, D. and T. C. Walther (2009). TORC2 plasma membrane localization is essential for cell viability and restricted to a distinct domain. *Mol Biol Cell* 20(5): 1565-1575.

Doris Berchtold performed all experiments, created all figures and contributed to writing the manuscript.

Publication II

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Doris Berchtold designed and performed all microscopy experiments and quantifications except for the membrane stretch experiment. She performed all yeast growth assays, phosphate-affinity gels, co-immunoprecipitations, and the anchor-away experiments. She designed and prepared all figures and co-wrote the manuscript.

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Berchtold, D., and T.C. Walther (2009). TORC2 plasma membrane localization is essential for cell viability and restricted to a distinct domain. *Mol Biol Cell*. 20: 1565-1575.