

Review

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Control of membrane fluidity: the OLE pathway in focus

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Abstract: The maintenance of a fluid lipid bilayer is key for membrane integrity and cell viability. We are only beginning to understand how eukaryotic cells sense and maintain the characteristic lipid compositions and bulk membrane properties of their organelles. One of the key factors determining membrane fluidity and phase behavior is the proportion of saturated and unsaturated acyl chains in membrane lipids. *Saccharomyces cerevisiae* is an ideal model organism to study the regulation of the lipid acyl chain composition via the OLE pathway. The OLE pathway comprises all steps involved in the regulated mobilization of the transcription factors Mga2 and Spt23 from the endoplasmic reticulum (ER), which then drive the expression of *OLE1* in the nucleus. *OLE1* encodes for the essential $\Delta 9$ -fatty acid desaturase Ole1 and is crucial for *de novo* biosynthesis of unsaturated fatty acids (UFAs) that are used as lipid building blocks. This review summarizes our current knowledge of the OLE pathway, the best-characterized, eukaryotic sense-and-control system regulating membrane lipid saturation, and identifies open questions to indicate future directions.

Keywords: Cdc48; ERAD; membrane fluidity; Mga2; OLE pathway; Spt23.

Introduction

Biological membranes are dynamic and complex assemblies of membrane lipids and proteins. The lipid

composition determines physicochemical membrane properties such as membrane thickness, bending rigidity, phase behavior and fluidity (Holthuis and Menon, 2014). The proportion of saturated and unsaturated lipid acyl chains is a key determinant of the molecular lipid packing and the resulting membrane fluidity (Ernst et al., 2016). Moreover, lipid saturation contributes to the identity of organelles of eukaryotes by determining their surface properties, which must be tightly controlled to maintain a functional secretory pathway (Preston et al., 2009; Payet et al., 2013). An increased proportion of saturated membrane lipids causes lipid bilayer stress and results in the activation of the unfolded protein response (UPR) (Deguil et al., 2011; Surma et al., 2013). Consequently, a failure to produce unsaturated membrane lipids can cause a severe reorganization of organelle abundance and morphology, and – in extreme cases – cell death (Zhang et al., 1999; Pineau et al., 2009; Preston et al., 2009; Hapala et al., 2011; Surma et al., 2013). The overproduction of unsaturated lipids, however, is equally harmful and can cause fatty acid-induced necrosis (Richly et al., 2005; Rockenfeller et al., 2010; Hapala et al., 2011; Eisenberg and Buettner, 2014; Ruggles et al., 2014). The best-characterized eukaryotic surveillance system of lipid saturation is the OLE pathway (Covino et al., 2016; Ernst et al., 2016). Intriguingly, the eukaryotic and prokaryotic sensing mechanisms differ significantly and have recently been compared and discussed (Ernst et al., 2016). By understanding the regulation of lipid saturation in *Saccharomyces cerevisiae*, we might be in a better position to identify new sensors in different organelles and organisms.

The baker's yeast *S. cerevisiae* has widely been used to study the architecture and regulation of lipid metabolism in eukaryotes. The lipid metabolic network, its branch points and regulation, and many biosynthetic enzymes are conserved from yeast to man (Lykidis, 2007; Henry et al., 2012). Both mammals and yeasts maintain membrane fluidity by generating CoA-activated, unsaturated fatty acids (UFAs) as lipid building blocks using a $\Delta 9$ -desaturase: the stearyl-CoA desaturase 1 (SCD-1) in mammals, and Ole1 in *S. cerevisiae*. Thus, Ole1 activity is essential for survival

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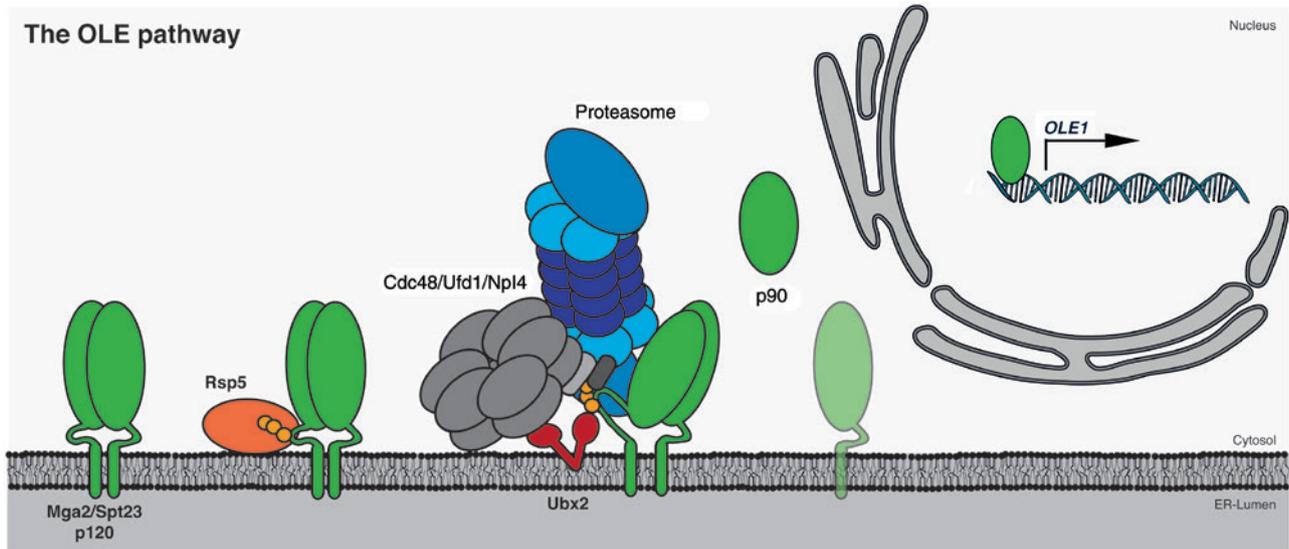


Figure 1: Schematic overview of the OLE pathway.

The transcriptional activators Spt23 and Mga2 are produced as membrane-tethered precursors (p120). For activation, they become ubiquitylated, processed by the proteasome, and mobilized from the membrane with the help of the Cdc48/Ufd1/Npl4 complex. The temporal order of these events is not well established. Once mobilized, the transcriptionally active p90 translocates to the nucleus to drive the expression of *OLE1* and other target genes.

of *S. cerevisiae* and tightly regulated via the OLE pathway (Figure 1) (Hoppe et al., 2000).

The OLE pathway regulates the activation and turnover of the transcriptional factors Mga2 and Spt23 (Zhang et al., 1997, 1999). These highly homologous transcription factors are produced as inactive, membrane-tethered precursors of ~120 kDa (p120). In order to generate a transcriptionally active fragment of ~90 kDa (p90), they need to be processed by the proteasome. After release from the ER-membrane, p90 can translocate to the nucleus and activate the transcription of *OLE1* and other targets (Hoppe et al., 2000; Piwko and Jentsch, 2006). Many components of the OLE pathway are shared with the ER-associated degradation (ERAD) pathway that removes misfolded proteins from the ER for terminal degradation in the cytosol (Raasi and Wolf, 2007; Vembar and Brodsky, 2008; Wolf and Stolz, 2012; Needham and Brodsky, 2013; Stordeur et al., 2014). Both the ERAD and OLE pathways rely on selective ubiquitylation by specific ubiquitin ligases, the segregase complex Cdc48/Ufd1/Npl4 (Hitchcock et al., 2001; Rape et al., 2001), its substrate recruiting factor Ubx2 (Neuber et al., 2005; Kolawa et al., 2013; Surma et al., 2013), the proteasome (Hoppe et al., 2001; Piwko and Jentsch, 2006), and a number of modulatory factors (Richly et al., 2005; Rumpf and Jentsch, 2006) (Figure 1). Notably, the identification of several key factors of the ubiquitin-proteasome

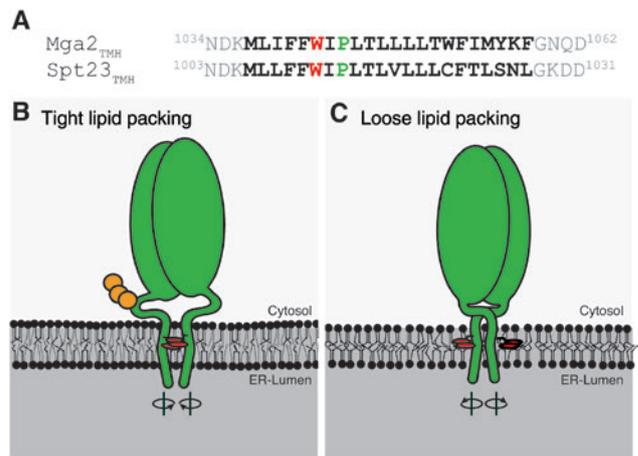


Figure 2: Membrane sensing by the OLE pathway.

(A) Sequence comparison of the TMH of Mga2 and Spt23, respectively. The sensory tryptophan residue is labeled red, a proline providing flexibility to the TMH is labeled green. (B) The dimeric transcription factors use their TMH to sense the molecular lipid packing in the ER-membrane. The TMHs explore alternative rotational orientations, but in the ER or growing yeast the bulky tryptophan residues (red) favor an orientation, which buries them in the dimer interface. This conformation is compatible with transcription factor ubiquitylation and activation (*ON*-conformation). (C) A high proportion of unsaturated lipids stabilizes an alternative rotational conformation of the TMHs, in which the bulky tryptophan residues (red) are rotated outwards toward the hydrophobic core of the membrane. This rotational orientation of the TMHs is incompatible with ubiquitylation (*OFF*-conformation).

system (UPS) by Stefan Jentsch and colleagues relied on the dramatic phenotypes associated with a deregulated UFA production in *S. cerevisiae* (Hoppe et al., 2000; Rape et al., 2001; Richly et al., 2005; Rumpf and Jentsch, 2006; Siepe and Jentsch, 2009). We could recently show that the homodimeric transcription factor Mga2 acts as a membrane sensor, whose conformation is controlled by the membrane environment (Covino et al., 2016) (Figure 2). The molecular lipid packing density in the core of the membrane determines the conformation in the trans-membrane region, which ultimately controls the ubiquitylation, proteolytic processing and mobilization of the transcriptionally active p90 (Figure 2). The temporal order of these downstream events, however, is challenging to study and remains controversially discussed. By summarizing available data and current models of the OLE pathway and by discussing the factors involved in processing and regulation (Figure 3), this review aims at identifying open questions to indicate future directions.

What is membrane fluidity and how to measure it?

‘Fluidity’ is defined as ‘the quality of a substance of being not solid and able to flow’ (Cambridge Advanced Learners Dictionary & Thesaurus) or ‘the ability of a substance to flow easily’ (Oxford Dictionary of English). According to the fluid mosaic model, a biological membrane is a two-dimensional fluid allowing lipids and proteins to diffuse freely in the plane of the membrane (Singer and Nicolson, 1972). Later, this model was extended to account for protein complexes, protein-lipid and lipid-lipid interactions as well as coexisting membrane domains with distinct fluidities (Nicolson, 2014). Nowadays, it is well accepted that biological membranes can form non-homogeneous, short-lived nano-domains that differ in their molecular constituents and nano-viscosity (Simons and Ikonen, 1997; Lingwood and Simons, 2010).

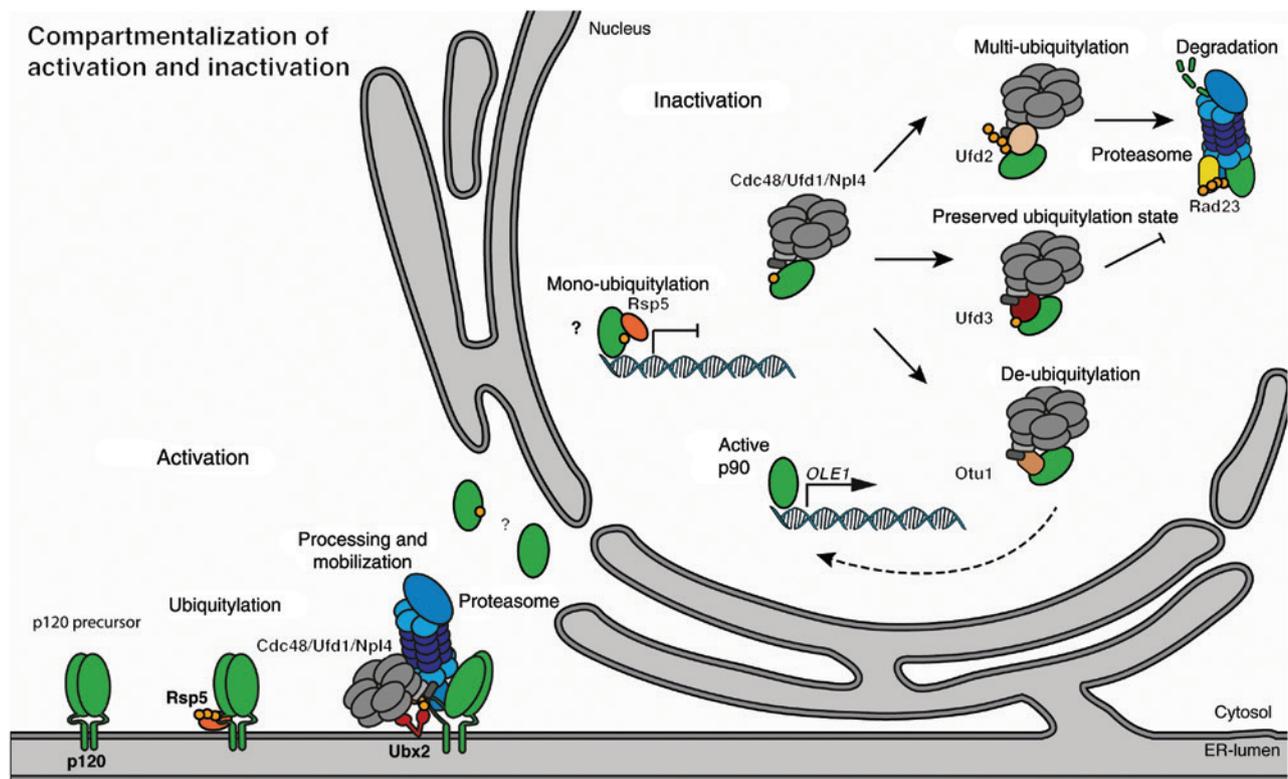


Figure 3: Activation and inactivation of the OLE pathway.

The dimeric p120 precursors are ubiquitylated (yellow circles) at the ER-membrane by the E3 ligase Rsp5, processed by the proteasome, and mobilized by Cdc48. After mobilization, p90 translocates to the nucleus to activate *OLE1* expression. Some factors required for activation, are also required for the degradation of p90 in the nucleus. Nuclear p90 might be ubiquitylated by Rsp5 and removed from the DNA by Cdc48/Ufd1/Npl4. Subsequent oligoubiquitylation by the E4 ligase Ufd2 and handover by Rad23 guide the transcription factor to the proteasome for terminal degradation. Ufd3 and Otu1 antagonize the degradation of nuclear p90: Ufd3 competes for the binding of Ufd2 to prevent oligoubiquitylation. Otu1 deubiquitylates p90 to recycle the transcription factor.

The bulk fluidity of a biological membrane can be described and measured by diverse spectroscopic methods, including electron paramagnetic resonance (EPR) and fluorescence spectroscopy. In the early 70s, there was already evidence for the lateral diffusion of spin-labeled lipids in the plane of a membrane (McConnell and Hubbell, 1971). Technically less demanding and much more frequently used, are fluorescence spectroscopic techniques relying on reporters, such as diphenylhexatriene (DPH), trimethylammonium diphenylhexatriene (TMA-DPH), or dyes of the laurdan series (Shinitzky et al., 1971; Shinitzky and Barenholz, 1974; Prendergast et al., 1981; Kaiser et al., 2009; Sezgin et al., 2014; do Canto et al., 2016). The tumbling rate of DPH and TMA-DPH is slowed down by increased membrane viscosity (i.e. decreased fluidity) causing fluorescence anisotropy (Lentz, 1989; Lande et al., 1995). The fluorescence emission spectrum of laurdan dyes reports on water penetration in the membrane bilayer due to molecular lipid packing defects that correlate with increased membrane fluidity (Kaiser et al., 2009). Biased by their structure and physicochemical properties, fluorescent reporters differ in their preferred position relative to the lipid bilayer and report on membrane fluidity from distinct regions of the membrane. Those that penetrate deep into the hydrophobic core are less sensitive to the lipid head group composition than those reporting from the membrane-water interface. EPR spin probes can be attached to different positions of lipid molecules to report the mobility of the probe and the polarity of its nano-environment. Systematic EPR studies with spin-labeled lipids revealed characteristic fluidity and polarity profiles across liposomal membranes of defined lipid compositions (McConnell and Hubbell, 1971; Marsh, 2001; Subczynski et al., 2010; Stepien et al., 2015). Thus, a biological membrane exhibits not just a single, bulk fluidity and its properties are not sufficiently described by a single number.

An exciting development is the increased use of molecular dynamics (MD) simulations to study the structure and properties of biological membranes (Smit et al., 1990; Marrink et al., 2007; Vattulainen and Rog, 2011). MD simulations provide access to short-lived phenomena at atomistic resolution allowing for a direct quantification of molecular packing densities, acyl chain order, lateral pressure profiles and lipid diffusion (Van Der Ploeg and Berendsen, 1982; Van Der Ploeg et al., 1983; Bennett and Tieleman, 2013). Because MD simulations at atomistic resolution are demanding with respect to computational time, it is currently not possible to characterize phenomena on a time scale much longer than $\sim 1 \mu\text{s}$ (Vattulainen and Rog, 2011; Marrink and Tieleman, 2013). Coarse

grained models such as the Martini model reduce the complexity at the cost of molecular detail and provide access to longer time scales and larger membrane systems (Marrink and Tieleman, 2013). However, only the combination of different techniques can provide holistic insight into the bilayer structure and its dynamics.

Membrane fluidity in the physiological context

Poikilothermic organisms such as bacteria, fungi, reptiles and fish cannot control their body temperature and must adapt their membrane lipid composition in order to maintain fluid membranes in the cold. Aided by mass spectrometry-based lipidomics, we are beginning to identify the sense-and-control systems orchestrating lipid metabolism during the homeoviscous adaptation and other forms of cellular stress (Sinensky, 1974; Hazel, 1995; Ernst et al., 2016). Eukaryotic cells face more complex challenges during adaptation than prokaryotes. The organelles of the secretory pathway differ in their lipid compositions with the molecular packing density and membrane rigidity increasing gradually from the ER toward the plasma membrane (van Meer et al., 2008; Holthuis and Menon, 2014). This gradient along the secretory pathway must be actively maintained during adaptive responses in order to warrant proper protein sorting and membrane trafficking. Surprisingly, little is known about the regulation of membrane fluidity in different organelles and especially in response to cellular stress. One of the reasons is that the tools established to study bulk membrane fluidity *in vitro* are often not applicable *in vivo* or limited to the plasma membrane. Sophisticated microscopic techniques combining stimulated emission depletion (STED) microscopy with single-molecule fluorescence-detection (Eggeling et al., 2009), and homo-fluorescence resonance energy transfer (Raghupathy et al., 2015) have been employed to study the dynamics and lateral organization of proteins and lipids in the plasma membrane, but not in other organelles. The lipid composition of intracellular membranes in *S. cerevisiae* have been studied after subcellular fractionation and/or immunoisolation (Zinser and Daum, 1995; Schneiter et al., 1999; Klemm et al., 2009). Given the critical role of these characteristic lipid compositions in determining organelle identity and function, it is clear that cells must collect information from the membranes of their organelles in order to adjust lipid metabolism during adaptive responses. Consequently, a number of cytosolic proteins with key regulatory roles in membrane trafficking

and lipid metabolism use amphipathic helices to explore the surface properties of cellular organelles to contribute to adaptive responses (Puth et al., 2015). Their mode of action has recently been discussed in excellent reviews (Bigay and Antonny, 2012; Cornell and Ridgway, 2015). Less is known about the mechanism of integral membrane sensors that are crucial to sense deep within the hydrophobic core of the lipid bilayer (Ernst et al., 2016). This review is dedicated to the mechanism and regulation of the OLE pathway that controls membrane fluidity in *S. cerevisiae*.

Mga2 and Spt23 as key regulators of membrane fluidity

The bakers yeast *S. cerevisiae* is an ideal model to study the regulation of membrane fluidity, because its genome encodes only a single fatty acid desaturase. Both the deletion and overexpression of the corresponding gene *OLE1* is lethal (Zhang et al., 1999). The desaturase is a heme-containing, ER-resident protein that requires molecular oxygen to introduce a double bond in CoA-activated saturated fatty acids (CoA-SFAs) to generate CoA-UFAs for glycerophospholipid biosynthesis (Stukey et al., 1989). The partially redundant transcription factors Mga2 and Spt23 control the expression of *OLE1*. Loss of both transcription factors is lethal within a few cell divisions unless UFAs are present in the growth medium, while single deletions are viable even in the absence of exogenous UFAs (Zhang et al., 1997). Homologs of Mga2 and Spt23 have been characterized in the fission yeast *Schizosaccharomyces pombe* and the pathogenic fungus *Candida albicans* (Oh and Martin, 2006; Burr et al., 2016). The remarkable sequence conservation of Mga2 and Spt23 among fungi including multiple pathogenic strains, and the absence of an obvious homolog in mammals makes these proteins intriguing drug targets for anti-fungal therapy.

A coordinated regulation of fatty acid desaturation and sterol biosynthesis is common to all fungi and mammals (Goldstein et al., 2006; Osborne and Espenshade, 2009; Raychaudhuri et al., 2012). The transcriptional regulation in mammals relies on sterol response element binding proteins (SREBPs), which have a fungal ancestor conserved in *S. pombe* but not in *S. cerevisiae* (Butler, 2013). A comparative characterization of the regulated activation of SREBPs and Mga2/Spt23 in different fungi paired with comprehensive transcriptome analyses might prove extremely informative (Raychaudhuri et al., 2012). It is tempting to speculate that the increased complexity of fatty acid desaturases in mammals or *Candida albicans*

compared to *S. cerevisiae* necessitates more complex networks of lipid and membrane sensors to coordinate fatty acid desaturation and sterol homeostasis (Oh and Martin, 2006). The simplistic OLE pathway of *S. cerevisiae* represents an ideal starting point for such an enterprise and is the main focus of this article.

Apart from *OLE1*, Mga2 and Spt23 control the expression of a number of highly expressed target genes involved in glucose and lipid metabolism, ribosome biogenesis and mating in *S. cerevisiae* (Auld et al., 2006). Although they do not contain classical DNA-binding domains, Mga2 and Spt23 control transcription through the fatty acid regulated (FAR) element (Choi et al., 1996) presumably by chromatin remodeling (Auld et al., 2006; Zhang et al., 1999). Nevertheless, Mga2 and Spt23 have different functions. Mga2, but not Spt23, activates the transcription of *ERG1* encoding for the squalene epoxidase of the ergosterol biosynthetic pathway (Rice et al., 2010). Likewise, Mga2 has been implicated in the adaptation to oxidative stress and the hypoxic response of *S. cerevisiae* by controlling ergosterol, zinc homeostasis, iron and ergosterol metabolism, and UFA production (Zhang et al., 1999; Jiang et al., 2001, 2002; Lyons et al., 2004; Kelley and Ideker, 2009).

The biosynthesis of ergosterol and UFAs is impaired by hypoxia, because Erg1 and Ole1 require molecular oxygen (Bloomfield and Bloch, 1960; Jahnke and Klein, 1983). A short phase of hypoxia cannot cause a significant remodeling of the composition of the ER membrane, as the effect of this transient perturbation is diluted in a large pool of ER lipids. However, prolonged periods of hypoxia do affect the membrane lipid composition of the ER and force the cells to react. The membrane-sensitive Mga2 is key to mount an adaptive response by sensing the shortage of ergosterol and unsaturated lipid in the ER-membrane and activating the expression of *ERG1* and *OLE1* (Zhang et al., 1999; Rice et al., 2010; Covino et al., 2016). In this light, the ER-membrane serves as ‘memory device’ to integrate a long-term signal for oxygen availability, which is encoded by the lipid composition (Covino et al., 2016; Ernst et al., 2016). This elegant mechanism of signal integration is apparently conserved among fungi, as the homolog of Mga2 in *S. pombe* was recently identified as oxygen-responsive modulator of lipid homeostasis (Burr et al., 2016).

Intra-membrane sensing by Mga2 controls the OLE pathway

Given the central role of unsaturated lipids for cellular growth and physiology it is clear that the OLE pathway

must be tightly controlled. In fact, when sufficient quantities of UFAs are available in the growth medium, the expression of *OLE1* ceases (Bossie and Martin, 1989; Stukey et al., 1989). Specific UFAs differ in their potential to attenuate *OLE1* expression and those with a cis- $\Delta 9$ double bond are most effective (McDonough et al., 1992). Based on these findings, a causal relationship between *OLE1* repression and the melting point of these UFAs had been suggested (Fujiwara et al., 1998). The observation that Spt23 and Mga2 exist as homodimeric, membrane-embedded precursors (Hoppe et al., 2000; Rape et al., 2001; Shcherbik and Haines, 2007), and that their proteolytic processing is controlled by dietary UFAs (Hoppe et al., 2000; Covino et al., 2016) paved the way toward the identification of the underlying molecular mechanism of regulation. Using a multi-disciplinary pipeline to study intra-membrane processes, we could show that Mga2 uses its transmembrane helix (TMH) to sense the proportion of unsaturated lipids in the ER-membrane (Covino et al., 2016) (Figure 2). Given the high sequence similarity of Mga2 and Spt23 in the sensory TMH region (86% sequence identity) (Figure 2A), it is likely that the activation of Spt23 is regulated via a similar mechanism.

Cellular growth relies on a constant supply of UFAs for membrane biogenesis. Consistently, the ER-membrane stabilizes an ubiquitylation-competent conformation of Mga2 that facilitates an efficient production of p90, which can be further improved when the level of saturated lipids in the ER-membrane increases (Figure 2B). As high proportions of unsaturated lipids impair membrane integrity, the expression of *OLE1* must cease when UFAs are taken up from the medium. Under these conditions, the ER-membrane stabilizes an alternative, ubiquitylation-incompetent conformation of Mga2 to block the proteolytic activation of Mga2 (Figure 2C).

The sensing mechanism of dimeric Mga2 involves dramatic rotational motions of the TMHs during which they establish a continuum of alternative TMH-TMH interfaces. Even minute changes in lipid saturation affect the population of alternative rotational conformations (Figure 2B, C). The conformation in the TMH region is propagated to the cytosolic portion of the protein, where Rsp5 mediates the ubiquitylation of Mga2 under normal growth conditions (*ON*-conformation) (Figure 2B). However, a membrane with a high proportion of unsaturated lipids stabilizes an alternative rotational orientation that blocks ubiquitylation and transcription factor activation (*OFF*-conformation) (Figure 2C). A bulky tryptophan residue located deep within the membrane turned out to be particularly crucial for the sensing mechanism based on TMH rotation (Figure 2A). EPR experiments and MD simulation revealed

that this residue is ‘hidden’ in the dimer interface, when the protein was situated in a membrane mimicking the acyl chain composition of the yeast ER (*ON*-conformation). However, when the molecular lipid packing in the core of the membrane decreases (e.g. due to dietary UFAs), the tryptophan residues rotate outwards toward the membrane environment (*OFF*-conformation) (Figure 2B). The membrane environment can trigger this dramatic conformational change, because the alternative rotational orientations are not separated by high-energy barriers. We propose that two features of the TMH are critical to support the sensitivity of the system (Figure 2A): (i) an interaction-prone TMH with aromatic residues distributed around the TMH, and (ii) a conserved proline residue that provides conformational flexibility to the TMHs during rotation and allowing for an intimate interaction of the two conserved tryptophans in the *ON*-conformation (Covino et al., 2016).

The equilibrium between alternative conformational and functional states is determined by the physicochemical properties of the dimeric TMHs and by the membrane environment. Consequently, it is possible to tune the OLE pathway by activating and inactivating mutations that shift the conformational equilibrium of the dimeric TMHs. However, the dose-response curve of inhibition by dietary UFAs is primarily determined by the membrane environment and less sensitive to mutations in the TMH. Thus, the regulation of the OLE pathway relies on a collective membrane property and not on a specific lipid-binding event. An obvious advantage of this mechanism is the inherent versatility. Even FAs such as linoleic acid (18 : 2) or arachidonic acid (20 : 4) that *S. cerevisiae* cannot synthesize, are incorporated into membrane lipids and contribute to a collective membrane property that controls the activity of the OLE pathway. Thus, *S. cerevisiae* can promiscuously take up a variety of FAs and use them for lipid biosynthesis, without losing control over membrane integrity. The particular sensitivity of the OLE pathway to UFAs with a double bond in the $\Delta 9$ position, however, suggests that the sensor was optimized by evolution to respond to endogenously produced UFAs.

Characterizing the OLE pathway – complications and challenges

The transcription factors of the OLE pathway are activated by a series of events that include their selective ubiquitylation, proteolytic processing by the proteasome, and the release of a transcriptionally active p90 that translocates into the nucleus (Figure 1). The temporal order

of these events remains unclear and partially controversial. With a few notable exceptions (Rape et al., 2001; Shcherbik and Haines, 2007; Covino et al., 2016) the OLE pathway has been studied by genetic means, which cannot unambiguously reveal the underlying molecular mechanisms. Several complications specific to the OLE pathway make it particularly challenging to draw a firm conclusion from genetic data. (i) Several components of the OLE pathway, especially those of the UPS, can interact with the transcription factors both in the cytosol and in the nucleus with opposing functions. The AAA-ATPase Cdc48, for example, has been implicated in the activation of the OLE pathway (Rape et al., 2001; Shcherbik and Haines, 2007), but also in its inactivation by facilitating the degradation of p90 in the nucleus (Richly et al., 2005) (Figure 3). This compartmentalization of activating and deactivating processes must be considered when the activity of the OLE pathway is assessed based on the steady-state levels of p120 and p90 in total cell lysates. (ii) The activity of the OLE pathway is controlled by the membrane lipid composition and the ambient temperature. While, temperature-sensitive mutants are extremely useful to study the function of essential gene products, it is crucial to realize that the necessary temperature shifts have an immediate effect on the activity of the OLE pathway by affecting physicochemical membrane properties. A similar consideration is important when using deletions of non-essential genes. All mutations that increase or decrease the activity of the OLE pathway have an impact on the lipid composition of the ER by modulating FA desaturation. These perturbed lipid compositions, however, directly affect the initiation of the OLE pathway and mount a homeostatic response. (iii) Membrane biogenesis and cell division are tightly connected to control organelle size and membrane abundance. Any manipulation affecting cellular growth, be it a switch of the carbon source to control the expression of a protein of interest or growth defect due to genetic manipulation, will affect the activity of the OLE pathway – directly and indirectly. (iv) Spt23 and Mga2 are relatively low abundant transcriptional activators. However, their activation, function, and turnover are often studied in the context of overexpression.

These complications make a straightforward interpretation of seemingly contradictory results extremely challenging. In order to address key mechanistic questions, the complex OLE pathway must be dissected into a series of sub-steps. Previous attempts to reconstitute individual steps of the OLE pathway with purified components (Rape et al., 2001; Shcherbik et al., 2003, 2004; Richly et al., 2005; Shcherbik and Haines, 2007; Bhattacharya et al.,

2009) hold great promise and should be intensified in the future using isolated forms of Mga2 and Spt23.

Ubiquitylation of Mga2 and Spt23

Protein ubiquitylation is mediated by an enzyme cascade, consisting of a ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and specific ubiquitin ligases (E3). Usually, ubiquitin is attached via its C-terminus to a lysine residue of a substrate molecule, leading to monoubiquitylation. The attachment of additional ubiquitin moieties to one of ubiquitin's seven lysine residues (K⁶, K¹¹, K²⁷, K²⁹, K³³, K⁴⁸ and K⁶³) leads to the formation of polymeric ubiquitin chains with different topologies. Ubiquitin chains can be branched or linear, with homogenous or mixed linkages (Komander and Rape, 2012). Deubiquitylating enzymes (DUBs) antagonize the ubiquitylation reaction and are involved in ubiquitin-chain remodeling. E4 enzymes are required for the synthesis of long multi-ubiquitin chains (Koegl et al., 1999). Length and the type of linkage of ubiquitin chains critically define the fate of substrate proteins. The best-characterized Lys⁴⁸-linked polyubiquitin chains are recognized by the proteasome and degraded. In contrast, Lys⁶³-linked ubiquitin chains and mono-ubiquitylation have been implicated in non-degradative processes, such as endocytosis, DNA-damage response and cell signaling (Komander et al., 2009; Saeki et al., 2009; Komander and Rape, 2012). Because of the versatile chain arrangements, it has been proposed that ubiquitin chains harbor a code to store and transmit information (Komander and Rape, 2012).

The ubiquitin ligase Rsp5 is essential for *S. cerevisiae*, and its loss can be partially rescued by supplementation of UFAs to the growth medium (Hoppe et al., 2000). This suggests an essential role of Rsp5 in the activation of the OLE pathway. In fact, both Mga2 and Spt23 contain a conserved binding motif (LPKY) for binding Rsp5 and their ubiquitylation is dependent on Rsp5 (Shcherbik et al., 2004). Because both the ubiquitylation and processing of Spt23 and Mga2 can be blocked by dietary UFAs, a signal must be transmitted from the sensory TMHs embedded in the ER-membrane to the site of ubiquitylation (Covino et al., 2016). With Rsp5 being stably associated with Spt23 and Mga2 (Shcherbik et al., 2002, 2003, 2004), it is conceivable that a *trans*-autoubiquitylation reaction, in analogy to the *trans*-autophosphorylation observed in receptor tyrosine kinases (Schlessinger, 2000), underlies the membrane-dependent ubiquitylation of Spt23 and Mga2 (Covino et al., 2016).

Several observations suggest that Rsp5-dependent ubiquitylation is required for the proteolytic processing of Spt23, but not Mga2 (Shcherbik et al., 2003, 2004; Shcherbik and Haines, 2007). In fact, a considerable degree of Mga2 p90 was observed in a *rsp5* deletion strain. Thus, ubiquitylation by Rsp5 is dispensable for the proteolytic processing of Mga2, but the subsequent release of the transcriptionally active p90 from membrane requires Rsp5 (Shcherbik et al., 2003).

Several questions regarding the ubiquitylation of Spt23 and Mga2 remain. (i) Which residues are ubiquitylated? (ii) Which type of ubiquitylation is used? (iii) What is encoded by the ubiquitylation signal? (iv) What is the role of ubiquitin-chain remodeling by DUBs and E4 ligases?

Three ubiquitylation sites have been identified in Mga2 and all map to the C-terminal portion of the protein that is rapidly degraded with the proteolytic activation. These residues K⁹⁸⁰, K⁹⁸³, and K⁹⁸⁵ are located in close proximity of the Rsp5 binding site (L⁹⁶⁷P⁹⁶⁸K⁹⁶⁹L⁹⁷⁰) (Bhattacharya et al., 2009). The polyubiquitin chain on Mga2 contains both K⁴⁸- and K⁶³-linked ubiquitin as evidenced by mass spectroscopy after immunoprecipitation (Saeki et al., 2009) and by *in vitro* ubiquitylation experiments using Rsp5 and mutant variants of ubiquitin (Shcherbik and Haines, 2007). The ubiquitylation sites of Spt23 remain ill-defined. It was reported that both p120 and p90 of Spt23 are ubiquitylated (Hoppe et al., 2000; Rape et al., 2001). Later, only the polyubiquitylation of Spt23 p120 was confirmed, while a mono- or di-ubiquitylation of p90 was not observed (Hoppe et al., 2000; Rape et al., 2001; Shcherbik and Haines, 2007; Siepe and Jentsch, 2009; Kolawa et al., 2013; Covino et al., 2016). It will be important to establish, whether the ubiquitylation of Spt23 and Mga2 differ significantly and how their stability is regulated in the nucleus.

In light of the published data it seems likely that polyubiquitylation at the ER-membrane is required for efficient release of the active transcription factor, and that a second ubiquitylation event might be required for the nuclear function of p90 and its degradation (Figure 3). In fact, Rsp5, Cdc48 and several of its cofactors affect the turnover of p90 and other proteins in the nucleus (see below; Figure 3).

Another layer of complexity was uncovered by the observation that the turnover of Spt23 is controlled by the prolyl isomerase Ess1 and that Spt23 p90 is phosphorylated (Siepe and Jentsch, 2009). Whether the phosphorylation of Spt23 occurs already at the ER-membrane or whether it occurs only in the nucleus, remains to be clarified. Now that the sensing mechanism of the OLE pathway is established at the molecular level (Covino et al., 2016),

it is time to characterize the signal propagation from the core of the membrane to the sites of ubiquitylation in order to resolve the molecular role of ubiquitylation for transcription factor processing and mobilization.

Transcription activation via proteasome-dependent processing

The proteolytic activation of a transcription factor is a recurring theme in the regulation of gene expression. Prominent examples are the sterol regulatory element binding proteins (SREBPs) that are translocated by the SREBP cleavage activation protein (SCAP) from the ER to the Golgi upon sterol depletion, where they are cleaved by the Site-1 and Site-2 proteases (Brown and Goldstein, 1997; Goldstein et al., 2006). The liberated N-terminal part migrates to the nucleus to activate the expression of target genes involved cholesterol biosynthesis and lipid metabolism including diverse fatty acid desaturases (Brown and Goldstein, 1997; Goldstein et al., 2006). In contrast to the proteolytic activation of Mga2, which is controlled by a collective membrane property (Covino et al., 2016), the regulated processing of SREBP-2 was reported to rely on a stereo-specific protein-lipid interaction (Radhakrishnan et al., 2004, 2008). The high-affinity binding of cholesterol to a specific loop of the tetrameric SCAP controls the interaction with SREBPs allowing for a switch-like regulation of the SREBP pathway (Radhakrishnan et al., 2004, 2008). Intriguingly, the activation of SREBP-1A and SREBP-1C can also be attenuated by UFAs in the medium (Hannah et al., 2001) and a specific interaction of free UFAs with UBXD8 was proposed to be critical for this regulation (Lee et al., 2010; Kim et al., 2013). Thus, mammalian cells that lack obvious homologs of Mga2 and Spt23 have established alternative regulatory mechanisms to adjust lipid metabolism in response to changes in the cellular content of sterols and UFAs. Whether or not mammalian cells use additional mechanisms to sense collective membrane properties remains to be rigorously tested.

The proteasome-dependent activation of Spt23 and Mga2 is initiated from a flexible loop between a tightly folded, N-terminal IPT domain (immunoglobulin-like/plexins/transcription factors; Mga P⁵³⁰-Q⁶¹⁰, Spt23 P⁵⁰⁸-N⁵⁸⁵) and two ankyrin repeats (Mga2 L⁷¹⁹-L⁷⁸¹, Spt23 R⁷⁰⁹-K⁷⁷¹). This loop is threaded into the proteolytic chamber of the proteasome to generate the p90 fragment and a second, short-lived C-terminal fragment (Hoppe et al., 2000; Piwko and Jentsch, 2006). The processive and complete degradation of p120 is prevented by the

tightly folded IPT domain that cannot enter the proteolytic chamber (Piwko and Jentsch, 2006). A mutant variant of Spt23 lacking the IPT domain (Spt23 Δ IPT) undergoes complete degradation and does not give rise to Spt23 p90 (Rape et al., 2001; Piwko and Jentsch, 2006). The steady-state level of Spt23 Δ IPT, however, was markedly increased by UFAs in the growth medium, showing that Spt23 Δ IPT is capable of membrane sensing and suggesting that dimerization via the IPT domain is not essential for proteasomal degradation *per se* (Rape et al., 2001; Piwko and Jentsch, 2006).

Currently, it is not entirely clear whether the proteolytic cleavage of Spt23 and Mga2 occurs spontaneously or whether it is assisted by an ubiquitylation event and the recruitment of the AAA-ATPase Cdc48 with its cofactors. The fate of the p120 precursors, however, their ubiquitylation and proteolytic processing, is unquestionable controlled by the membrane environment (Hoppe et al., 2000; Covino et al., 2016).

The role of the Cdc48 complex in the OLE pathway

The highly conserved AAA-ATPase Cdc48 (VCP or p97 in mammals) has been implicated in diverse cellular pathways, including membrane fusion, mitotic spindle disassembly, mitochondria-associated degradation (MAD) and ERAD (Latterich et al., 1995; Xu et al., 2011; Wolf and Stolz, 2012). In order to fulfill these functions, Cdc48 associates

with a large number of substrate-adaptors and cofactors. The heterodimeric Ufd1/Npl4 cofactor binds ubiquitin-chains and functions in several ubiquitin-proteasome dependent pathways (Richly et al., 2005) including the OLE and the ERAD pathways. Another family of cofactors is characterized by a diagnostic UBX domain that binds to the N-terminal region of Cdc48 (Neuber et al., 2005; Schubert and Buchberger, 2008; Wang and Lee, 2012; Kolawa et al., 2013). This family has seven members in *S. cerevisiae* and each of these recruits Cdc48 to different cellular locations to fulfill its function. Ubx2 acts as membrane anchor for Cdc48 in the ER and uses an UBA domain to bind and recruit ubiquitylated substrates to Cdc48 in the ERAD and OLE pathways (Neuber et al., 2005; Schubert and Buchberger, 2005; Surma et al., 2013).

The mechanism and precise entry-point of Cdc48 in the OLE pathway remains to be established. Based on the available data, there are at least two possibilities how Cdc48 could contribute to the activation of Mga2 and Spt23. The first possibility is that the proteolytic processing of Spt23 and Mga2 is completely independent of Cdc48. In this scenario the AAA-ATPase acts only after processing as a segregase to release the processed p90 form from its unprocessed and membrane-embedded interaction partner (Figure 4A) (Rape et al., 2001; Shcherbik and Haines, 2007). The necessary pulling-force would be provided by ATP-hydrolysis and concomitant conformational changes of the AAA-ATPase (Xia et al., 2016). The transcriptionally active p90 form of Spt23 might be released from the ER as monoubiquitylated species (Rape et al., 2001). Mga2 in contrast, is thought to be mobilized

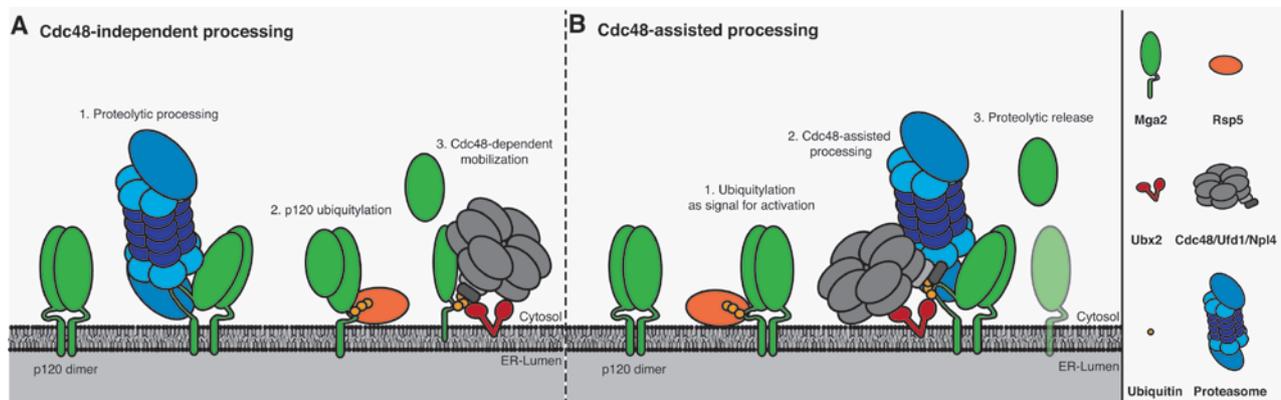


Figure 4: The unknown role of Cdc48.

(A) Cdc48-independent processing. According to this hypothetical model, the transcription factors are processed by the proteasome fully independently of Cdc48. After processing, p90 remains tightly attached to its unprocessed partner. The Cdc48/Ufd1/Npl4 complex is required to remodel this complex and to release p90. (B) Cdc48-assisted processing. According to this hypothetical model the activity of the Cdc48/Ufd1/Npl4 complex is required for proteasomal processing and degradation. By removing the membrane anchor and/or full degradation of the interacting p120, processed p90 is released from the ER-membrane by the joint activity of the Cdc48/Ufd1/Npl4 complex and the proteasome.

as unmodified p90 that was liberated from a polyubiquitylated p120 interaction partner by ATP-dependent remodeling of the p120:p90 complex (Shcherbik and Haines, 2007). It is not entirely clear, however, which handle Cdc48 would use to remove a processed and non-modified p90 from its membrane-bound interaction partner.

The second scenario suggests that the prime function of Cdc48 and its cofactors is to facilitate a rapid proteolysis of the C-terminal portion of p120 (Figure 4B) (Hitchcock et al., 2001; Raasi and Wolf, 2007; Kolawa et al., 2013). This would be reminiscent of its role in the ERAD pathway (Vembar and Brodsky, 2008). Supporting evidence is that interference with the Cdc48/Ufd1/Npl4 complex at the ER-membrane leads to an accumulation of ubiquitylated and unprocessed forms of Spt23 and Mga2 suggesting a retardation of processing (Hitchcock et al., 2001; Rape et al., 2001; Kolawa et al., 2013; Surma et al., 2013). Intriguingly, the oligomeric state of the mobilized p90 has never been studied. A proteolysis of both C-terminal portions from a dimeric p120:p120 precursor, would mobilize a p90:p90 dimer that might be stabilized by the IPT domain. A variation of this theme would be the release of a single p90 by a rapid and complete degradation of the interacting, ubiquitylated p120 interaction partner (Shcherbik et al., 2003; Shcherbik and Haines, 2007). Based on published observations with apparently contradicting observations, we can neither prove nor disprove the validity of these models. Only the reconstitution of the OLE pathway with purified components might shed new light on critical requirements for transcription factor activation and establish the sequence reactions.

Once the transcriptionally active p90 of Mga2 and Spt23 are mobilized from the membrane, they enter the nucleus to drive the expression of *OLE1* and other target genes. Here, Rsp5 and Cdc48 with its binding partners Ufd2, Rad23, and Dsk2 are required for the proteasomal degradation of Spt23 p90 (Richly et al., 2005; Rumpf and Jentsch, 2006). Ufd2 represents an E4 ubiquitin ligase promoting the multiubiquitylation of Cdc48-associated proteins, while Rad2, and Dsk2 act as shuttle factors handing over ubiquitylated proteins from Cdc48 to the nuclear proteasome (Richly et al., 2005) (Figure 3). The turnover of p90 in the nucleus is antagonized by the Cdc48 binding protein Ufd3 (Doa1) and the deubiquitylating enzyme Otu1 binding to the N-terminal domains of the hexameric Cdc48 (Figure 3) (Rumpf and Jentsch, 2006). Thus, several nuclear factors contribute to the activity of the OLE pathway by affecting the turnover of Spt23 p90. They are, however, not specific to the OLE pathway and have general functions for the UPS. Otu1, for example, is

conserved from yeast to man and involved in the removal of misfolded proteins from the ER (Ernst et al., 2009; Stein et al., 2014). Cdc48 has at least a dual function by mobilizing p90 from the ER to activate the OLE pathway and by mediating its turnover in the nucleus.

Future perspectives and concluding remarks

In this review, we have summarized our current knowledge of the OLE pathway and have identified a number of unresolved questions. The architecture of the OLE pathway with an in-built feedback control via the membrane lipid composition makes a mechanistic understanding of the pathway based on genetic experiments extremely challenging. Only the dissection of the OLE pathway into smaller steps and their reconstitution with purified components will establish the temporal order of events that lead to transcription factor activation.

An important open question addresses the role of crosstalk between the transcriptional activators of the OLE pathway. Spt23 and Mga2 have distinct cellular functions and target genes. Given the high overall sequence identity of 39% it seems likely that their mode of activation and regulation is similar. However, whether Spt23 uses a similar rotation-based mechanism of membrane sensing as Mga2 remains to be vigorously tested (Covino et al., 2016). Mga2 is the dominant factor contributing to *OLE1* expression and key to the response to hypoxia, cobalt, nickel, and oxidative stress (Chellappa et al., 2001; Jiang et al., 2001; Vasconcelles et al., 2001; Kelley and Ideker, 2009). Future work shall establish, whether Mga2 serves as the main regulator of the OLE pathway, while Spt23 is responsible only for fine-tuning. Another intriguing possibility is that Spt23 and Mga2 might form heterodimers. It is well accepted that Mga2 and Spt23 form homodimers that are stabilized by the TMH and the cytosolic IPT domains (Rape et al., 2001; Covino et al., 2016). Given the high sequence similarity in these regions, it is tempting to speculate that Spt23 and Mga2 might form heterooligomers. In fact, there is genetic evidence to support this possibility. The expression of a soluble variant of Mga2 lacking its membrane anchor is sufficient to induce the expression of *OLE1*. Somewhat surprisingly, the induction of *OLE1* by this construct is sensitive to dietary UFAs, but only in cells lacking *SPT23* (Chellappa et al., 2001). This interdependence of Spt23- and Mga2-dependent transcriptional regulation of *OLE1* might point at a direct interaction of these factors.

The OLE pathway uses a remarkable sensing mechanism to control the expression of *OLE1* and to control the biosynthesis of unsaturated lipids in *S. cerevisiae*. Mga2 and Spt23, however, are not conserved in metazoans. How do metazoans sense and control the acyl chain composition in the ER? How do other organelles sense and maintain their unique properties? How do highly specialized cells with unique membrane lipid composition such as glia cells, neurons, or photoreceptor cells control their lipid metabolism in order to establish membranes with unique properties? We are convinced that a large number of membrane sensors and regulatory mechanisms are waiting for their identification.

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