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FapR: From Control of Membrane Lipid Homeostasis to a Biotechnological Tool

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Phospholipids and fatty acids are not only one of the major components of cell membranes but also important metabolic intermediates in bacteria. Since the fatty acid biosynthetic pathway is essential and energetically expensive, organisms have developed a diversity of homeostatic mechanisms to fine-tune the concentration of lipids at particular levels. FapR is the first global regulator of lipid synthesis discovered in bacteria and is largely conserved in Gram-positive organisms including important human pathogens, such as *Staphylococcus aureus, Bacillus anthracis,* and *Listeria monocytogenes*. FapR is a transcription factor that negatively controls the expression of several genes of the fatty acid and phospholipid biosynthesis and was first identified in *Bacillus subtilis*. This review focuses on the genetic, biochemical and structural advances that led to a detailed understanding of lipid homeostasis control by FapR providing unique opportunities to learn how Gram-positive bacteria monitor the status of fatty acid biosynthesis and adjust the lipid synthesis accordingly. Furthermore, we also cover the potential of the FapR system as a target for new drugs against Gram-positive bacteria as well as its recent biotechnological applications in diverse organisms.

Keywords: lipid synthesis, FapR, transcriptional regulation, Gram-positive bacteria, *in vivo* malonyl-CoA sensor, synthetic biology

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INTRODUCTION

The cell membrane, consisting mainly of a fluid phospholipid bilayer in which a variety of proteins are embedded, is an essential structure to bacteria making membrane lipid homeostasis a crucial aspect of bacterial cell physiology. The production of phospholipids requires of the biosynthesis of fatty acids and their subsequent delivery to the membrane-bound glycerol-phosphate acyltransferases. In all organisms fatty acids are synthetized via a repeated cycle of reactions involving the condensation, reduction, hydration, and reduction of carbon-carbon bonds (Rock and Cronan, 1996; Campbell and Cronan, 2001). In mammals and other higher eukaryotes, these reactions are all catalyzed by a large multifunctional protein, known as type I synthase (FAS I), in which the growing fatty acid chain is covalently attached to the protein (Rock and Cronan, 1996; Campbell and Cronan, 2001). In contrast, bacteria, plant chloroplasts, and *Plasmodium falciparum* contain a type II system (FAS II) in which each reaction is catalyzed by a discrete protein. A characteristic of FASII is that all fatty acyl intermediates are covalently connected to a small acidic protein named acyl carrier protein (ACP), and sequentially shuttled from one enzyme to another. A key molecule for fatty acid elongation is malonyl—coenzyme A (CoA) which is formed by

carboxylation of acetyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) (**Figure 1**). This biosynthetic scheme is conserved in all fatty acid producing bacteria, but the substrate specificity of some of the enzymes involved in the pathway leads to the variety of fatty acids found in different bacterial genera (Campbell and Cronan, 2001; Lu et al., 2004). When the acyl-ACPs reach the proper length they become substrates for the acyltransferases that transfer successively the fatty acyl chains into glycerol phosphate to synthetize phosphatidic acid (PtdOH), the universal intermediate in the biosynthesis of membrane glycerophospholipids (**Figure 1**: Campbell and Cronan, 2001; Rock and Jackowski, 2002). There are two enzyme systems that carry out the first transacylation reaction in bacteria. In the first one, present exclusively in Gram-negative bacteria (primarily gamma-proteobacteria), either acyl-ACP or acyl-CoA thioesters are utilized by the membrane-bound PlsB acyltransferase to acylate position 1 of glycerol-P giving 1-acylglycerol phosphate (Parsons and Rock, 2013). The second enzyme system, widely distributed and predominating in Gram-positive bacteria, consist of the PlsX/Y pathway for 1-acyl-glycerol phosphate formation (Lu et al., 2006; Schujman and de Mendoza, 2006; Paoletti et al., 2007). PlsX is a membrane associated protein (Sastre et al., 2016) that catalyzes the formation of a novel acyl donor, acyl phosphate (acyl-P), from acyl-ACP. This activated fatty acid is then used by the membrane-bound PlsY acyl transferase to acylate the position 1 of glycerol phosphate. The PlsX/PlsY system is also present in *E. coli* although its precise role is still an enigma as *plsB* is an essential gene in this bacterium (Parsons and Rock, 2013). Independently of the first enzyme system used, the second acyl transferase in PtdOH formation is PlsC, which is universally expressed in bacteria. This enzyme completes the synthesis of PtdOH by transferring an acyl chain to the position 2 of 1-acyl-glycerol phosphate. In the case of Gram-positive bacteria, PlsC isoforms exclusively utilize acyl-ACP (Lu et al., 2006; Paoletti et al., 2007), while *E. coli* PlsC can use both, acyl-ACP or acyl-CoA, as substrates (Coleman, 1992).

The fluidity of the lipid bilayer is essential for the normal function of the cellular membrane and bacteria normally control its physical state by modifying the incorporation of a mixture of fatty acids with different melting temperatures into phospholipids. In this sense, many bacteria respond to a decrease in temperature, which increases membrane rigidity, by increasing the proportion of unsaturated fatty acids (UFAs) into the phospholipids and viceversa (Zhang and Rock, 2008). Unsaturated double bonds in lipids generate kinks into the otherwise straightened acyl hydrocarbon chain and thereby increase membrane fluidity. Hence, the production of UFAs and its regulation are important processes in membrane homeostasis in bacteria and the underlying diverse mechanisms have been recently revised elsewhere (Mansilla et al., 2008; Parsons and Rock, 2013).

Due to the fact that the membrane lipid bilayer is an essential structure for every living cell and its biogenesis implies a high energetic cost, mainly due to fatty acid biosynthesis, organisms have developed a variety of homeostatic mechanisms to finely adjust the concentration of lipids at particular levels. Bacteria possess regulatory mechanisms acting directly on the activities of the lipid biosynthetic enzymes, but have also evolved sophisticated mechanisms to exert an exquisite control over the expression of the genes involved in lipid metabolism (Zhang and Rock, 2008; Parsons and Rock, 2013). Six transcriptional regulators controlling the expression of genes involved in fatty acid biosynthesis have been identified to date in bacteria. Among them, FadR (Henry and Cronan, 1991, 1992; Lu et al., 2004), DesR (Aguilar et al., 2001; Mansilla and de Mendoza, 2005), FabR (Zhang et al., 2002), and DesT (Zhu et al., 2006; Zhang et al., 2007), are committed to adjust unsaturated fatty acids to proper levels in membrane phospholipids while FapR (Schujman et al., 2003) and FabT (Lu and Rock, 2006) are global transcriptional repressors in Gram-positive bacteria that simultaneously regulate the expression of a number of genes involved in fatty acid and phospholipid metabolism.

This review focuses on the genetic, biochemical and structural characterization of FapR which paved the way to a major advance in our understanding of the molecular basis of the lipid homeostasis control in bacteria. We will also cover the potential of this regulatory system as a target for new antibacterial compounds as well as emerging biotechnological applications based on it.

THE DISCOVERY OF THE FapR SYSTEM

FapR from *Bacillus subtilis* was the first global transcriptional regulator of FASII to be discovered in bacteria (Schujman et al., 2003). The initial evidence that fatty acid biosynthesis was transcriptionally regulated came from the study of *lacZ* fusions to the promoter region of the *fabHAF* operon of *B. subtilis*, which codes for two key enzymes involved in the elongation of fatty acids (Schujman et al., 2001). These studies showed that the operon *fabHAF* is transcribed during exponential phase but when the cell culture approaches to stationary phase its transcription is turned off (Schujman et al., 2001). This finding is consistent with the observation that during exponential growth bacteria constantly produce new membrane in order to divide and hence need to actively synthetize fatty acids. Nevertheless, when cell division is completed membrane growth stops and fatty acid synthesis is turned off. An important finding was that when fatty acid synthesis is inhibited the transcription of the *fabHAF* operon is induced with the concomitant increment in protein levels (Schujman et al., 2001). Thus, it was proposed that *B. subtilis* is able to detect a decrease in the activity of FASII and respond accordingly by inducing the production of the condensing enzymes FabHA and FabF (Schujman et al., 2001). Moreover, DNA microarray studies indicated that upon inhibition of fatty acid synthesis the transcription of ten genes was induced (Schujman et al., 2003). These genes coded for proteins involved in fatty acid and phospholipid biosynthesis and belonged to six operons (the *fap* regulon) (Schujman et al., 2003). Furthermore, a conserved 17 bp inverted repeat within, or immediately downstream, of the *fap* predicted promoters, consistent with a putative binding site for a transcriptional repressor, was identified (Schujman et al., 2003). The corresponding binding protein

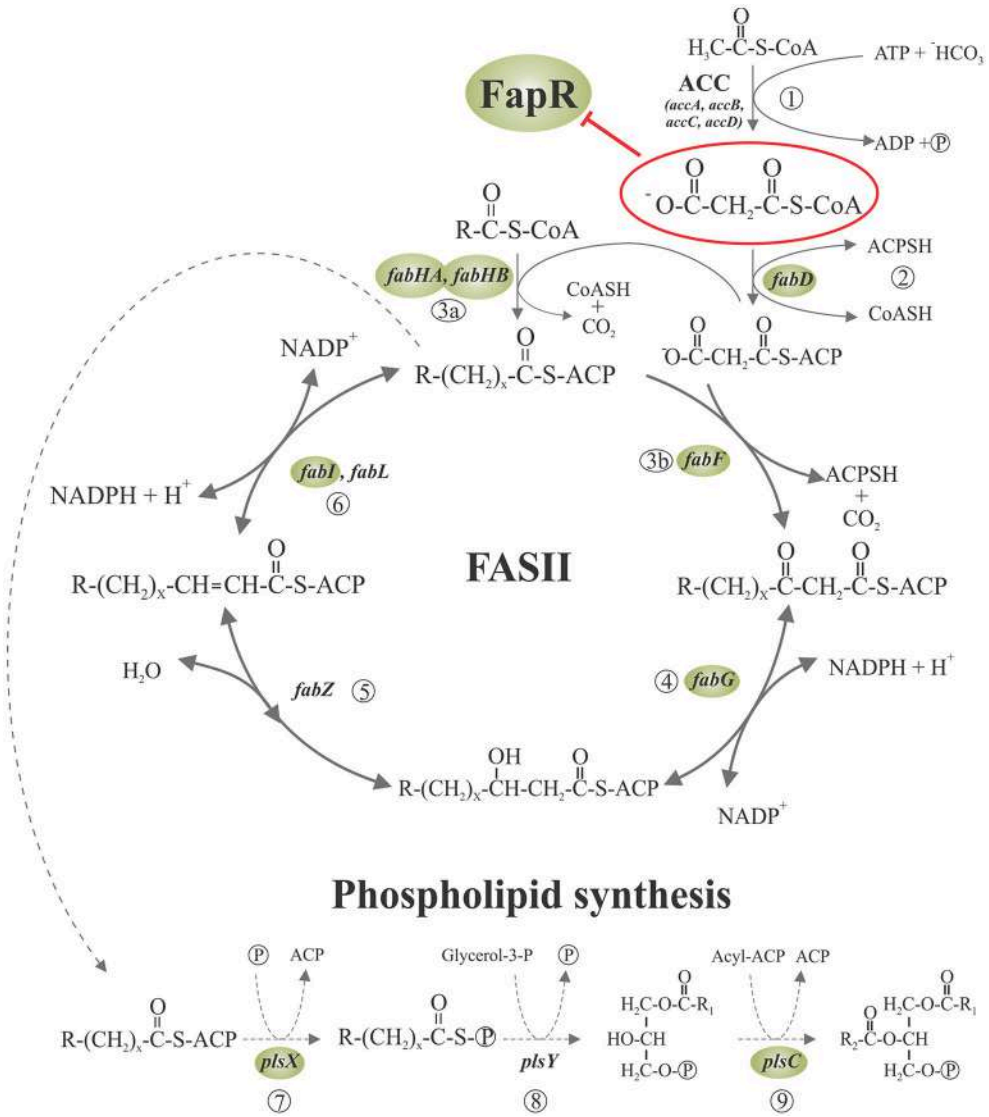

FIGURE 1 | Fatty acid synthesis and phospholipid initiation steps in *Bacillus subtilis*. Malonyl-CoA is generated from acetyl-CoA by acetyl-CoA carboxylase (ACC) (1) and then is transferred to ACP by malonyl-CoA transacylase (2). The FabH condensing enzymes initiates the cycles of fatty acid elongation by condensation of acyl-CoA primers with malonyl-ACP (3a). The resultant β-ketoester is reduced by the β-ketoacyl-ACP reductase (4). Then, the β-hydroxyacyl-ACP is dehydrated to the trans-2 unsaturated acyl-ACP by β-hydroxyacyl-ACP dehydrase (5), which is finally reduced by enoyl reductase (6). Subsequent rounds of elongation are initiated by the elongation-condensing enzyme FabF (3b) to generate an acyl-ACP two carbons longer than the original acyl-ACP at the end of each cycle. The long chain acyl-ACP end products of fatty acid synthesis are transacylated in three steps to glycerolphosphate, to generate phosphatidic acid (PA), a key intermediate in the synthesis of phospholipids. First, PlsX catalyzes the synthesis of fatty acyl-phosphate from acyl-ACP (7); then, PlsY transfers the fatty acid from the activated acyl intermediate to the 1-position of glycerol-3-phosphate (8) and finally, lyso-PA is acylated to PA by PlsC (9). Expression of the genes surrounded by shaded ellipses is repressed by the transcriptional regulator FapR, whose activity is, in turn, antagonized by malonyl-CoA (enclosed in a red ellipse). R denotes the terminal group of branched-chain or straight-chain fatty acids. Adapted from Albanesi et al. (2013).

was isolated from cells extracts using a DNA fragment carrying the promoter region of *fabHA* and identified by N-terminal sequencing (Schujman et al., 2003). The gene encoding the global transcriptional repressor was named *fapR* for *fatty acid and phospholipid regulator* (Schujman et al., 2003). The binding of FapR to the promoter regions of the regulated genes, and its dependence on the 17 inverted repeats was demonstrated *in vitro*. It was also showed that in a *fapR* null mutant the expression of the *fap* regulon is upregulated and that this expression is not further increased upon inhibition of FASII (Schujman et al., 2003). Therefore, it was established that FapR was a novel global negative regulator of lipid biosynthesis in Gram-positive bacteria and that FapR was involved in the observed induction of transcription in the presence of fatty acids synthesis inhibitors (Schujman et al., 2003). Bioinformatic analyses indicated that FapR is present and highly conserved in all the species of

the *Bacillus, Listeria,* and *Staphylococcus* genera (all including important human pathogens like *Bacillus anthracis*, *Bacillus cereus, Listeria monocytogenes,* and *Staphylococcus aureus*) as well as in the pathogen *Clostridium difficile* and other related genera. However, *fapR* was not found in Gram-negative bacteria or other Gram-positive genera (Schujman et al., 2003). Furthermore, in the bacterial species bearing FapR, the consensus binding sequence for the repressor is also highly conserved in the putative *fapR* promoter region. Altogether, the observations suggested that the regulatory mechanism identified in *B. subtilis* could be conserved in many other bacteria (Schujman et al., 2003). Indeed, genetic and biochemical assays proved this is the case in *S. aureus* (Albanesi et al., 2013).

MALONYL-CoA: THE EFFECTOR MOLECULE

A central question in the regulation of the *fap* regulon by FapR was how the status of fatty acids synthesis controlled the activity of the repressor. The fact that (i) the *acc* genes, encoding the subunits of the acetyl-CoA carboxylase (ACC), which catalyzes the synthesis of malonyl-CoA (**Figure 1**), are not under FapR control (Schujman et al., 2003), (ii) malonyl-CoA concentrations are known to increase upon inhibition of fatty acid synthesis (Heath and Rock, 1995), and (iii) the only known fate of malonyl-CoA in *B. subtilis* and most other bacteria is fatty acid synthesis (James and Cronan, 2003), pointed to malonyl-CoA as a reasonable candidate to be the regulatory ligand. Two observations gave experimental support to this hypothesis. First, expression of the *fap* regulon was derepressed by antibiotics that inhibit fatty acid biosynthesis with the concomitant increase in the intracellular levels of malonyl-CoA (Schujman et al., 2001). Second, this upregulation was abolished by precluding the transcription of genes encoding the subunits of the acetyl-CoA carboxylase (ACC) (Schujman et al., 2003).

A key issue was to establish if malonyl-CoA bound directly to FapR to regulate its activity or if it was first converted into another product that acted as a signaling molecule. The finding that antibiotics against different steps of FASII led to the transcriptional induction of the *fap* regulon, even when the *B. subtilis fabD* gene (Morbidoni et al., 1996) was not expressed, suggested that malonyl-CoA could be the direct effector of FapR (Schujman et al., 2003). FabD converts malonyl-CoA into malonyl-ACP, which, in turn, is only utilized in the elongation of fatty acid synthesis (de Mendoza et al., 2002). *In vitro* transcription experiments from several promoters of the *fap* regulon, including the *fapR*-operon promoter (P*fapR*), proved that FapR is unable to repress transcription in the presence of malonyl-CoA. Moreover, these assays showed that this molecule operates not only as a direct but also as a specific inducer of the *fap* promoters since different acyl-CoA derivatives related to malonyl-CoA (such as acetyl-CoA, propionyl-CoA, succinyl-CoA, and butyryl-CoA), were not able to prevent FapR transcriptional repression (Schujman et al., 2003). The same direct and specific role of malonyl-CoA as the effector molecule was shown for FapR of *S. aureus* (*Sa*FapR) (Albanesi et al., 2013).

STRUCTURAL SNAPSHOTS OF THE FapR REGULATION CYCLE

Like many transcriptional regulators in bacteria, FapR is a two-domain protein with an N-terminal DNA-binding domain (DBD) connected through a linker α-helix (αL) to a larger C-terminal effector-binding domain (EBD) (Schujman et al., 2006). The first insights on the molecular mechanism for the control of FapR activity came from the crystal structures of truncated forms of FapR from *B. subtilis* (*Bs*FapR) (Schujman et al., 2006). These structures showed that the EBD is a symmetric dimer displaying a "hot-dog" architecture, with two central α-helices surrounded by an extended twelve-stranded β-sheet (Schujman et al., 2006). This fold is similar to the one observed in many homodimeric acyl-CoA-binding enzymes (Leesong et al., 1996; Li et al., 2000) involved in fatty acid biosynthesis and metabolism (Dillon and Bateman, 2004; Pidugu et al., 2009). Interestingly, FapR, a bacterial transcriptional repressor, seems to be the only well-characterized protein to date with no-enzymatic function that harbors the "hot-dog" fold (Albanesi et al., 2013). On the other hand, the EBD domain of *Bs*FapR was crystallized in complex with malonyl-CoA. Comparison of both structures revealed structural changes induced by the effector molecule in some ligand-binding loops of the EBD that were suggested to propagate to the N-terminal DBDs impairing their productive association for DNA binding (Schujman et al., 2006). However, the actual mechanisms involved in the regulation of FapR activity remained largely unknown due to the lack of detailed structural information of the full-length repressor and its complex with DNA. Recently, important mechanistic advances into the mode of action of FapR were done through the structural characterization of the full-length repressor from *S. aureus* (*Sa*FapR). The crystal structures of *Sa*FapR were obtained for the protein alone (apo-*Sa*FapR) as well as in complex with the cognate DNA operator and the effector molecule malonyl-CoA (Albanesi et al., 2013) (**Figure 2**).

Structure of the *Sa*FapR-DNA Complex

The crystal structure of the *Sa*FapR-DNA complex was obtained using a 40-bp oligonucleotide comprising the P*fapR* promoter, which, as mentioned above, belongs to the *fap* regulon (Schujman et al., 2006). In the crystal, two *Sa*FapR homodimers were observed to bind to each DNA molecule. Interestingly, an inverted repeat covering half of the FapR-protected region in DNAseI footprinting analyses (Schujman et al., 2006), corresponded to the recognition site of one of the homodimers (Albanesi et al., 2013). This suggested a sequential mechanism of binding that was confirmed by isothermal titration calorimetry (ITC) studies of the *Sa*FapR-DNA interaction, which also provided the dissociation constants of each binding reaction (Albanesi et al., 2013). In the crystal structure of the *Sa*FapR-DNA complex, each protein homodimer exhibited an elongated asymmetric conformation with the two DNA-bound DBDs completely detached from the central dimeric "hot-dog" EBD (**Figure 2A**) (Albanesi et al., 2013). In each homodimer the amphipatic linker α-helixes from the protomers (αL and αL′) interact, mainly through their exposed hydrophobic faces,

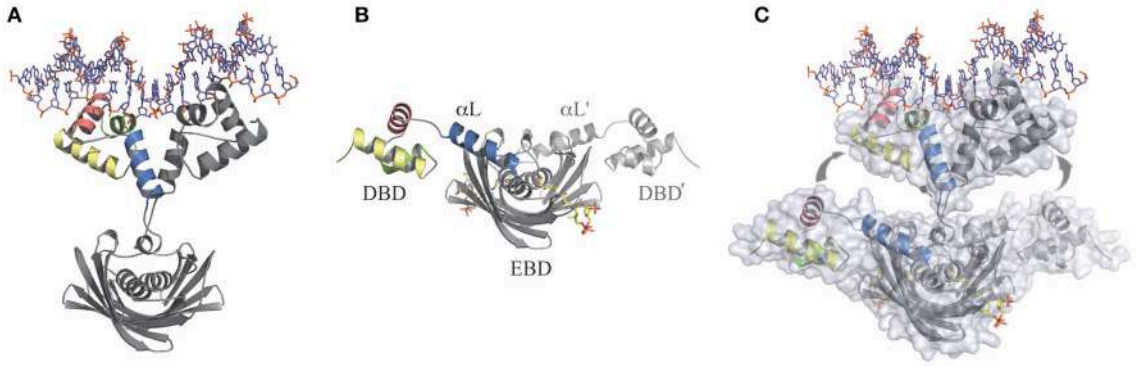

FIGURE 2 | The transitional switch between the relaxed and tense states of FapR involves a significant structural rearrangement of the DBDs. **(A)** Relaxed state: FapR in complex with DNA in which the amphipathic linker α-helix (αL) from each protomer associates with each other. **(B)** Tense state: FapR in complex with malonyl-CoA (shown in stick representation). **(C)** Superposition of the two conformational states of the repressor illustrating the structural transition which involves substantial changes and large (∼30 Å) inter-domain movements. Solvent accessible surfaces are shown in transparent to highlight the DNA-induced dissociation of the invariant effector-binding domain (EBD) from the DNA-binding domains (DBDs). The molecules are shown in light (relaxed) and dark (tense) gray, except for the helices from one DBD (colored). Adapted from Albanesi et al. (2013).

playing an important role in the stabilization of *Sa*FapR's molecular architecture in the complex with DNA (**Figure 2A**) (Albanesi et al., 2013). On their hand, both DBDs interact in a similar manner with DNA establishing sequence-specific contacts between the helix-turn-helix motifs with the major and minor grooves of the DNA double helix (Albanesi et al., 2013). Importantly, two arginine residues from each linker αL (one from αL and one from αL′) make base-specific interactions in the minor groove promoting its opening and inducing a pronounced local bending of DNA (Albanesi et al., 2013). Notably, the aminoacid residues making key contacts with DNA are highly conserved in FapR from all bacterial species where it was identified indicating the DNA-binding-mode of this transcriptional repressor is conserved (Albanesi et al., 2013).

Structure of the *Sa*FapR-Malonyl-CoA Complex

The crystal structure of full-length *Sa*FapR in complex with malonyl-CoA showed that in the presence of the effector molecule the repressor adopts a quaternary arrangement that is different and more compact than when bound to DNA (**Figure 2B**) (Albanesi et al., 2013). In this conformation, both amphipathic linker helices αL bind to either side of the central EBD domain instead of interacting with each other as when binding DNA. Like this, the two DBDs domain are far apart from each other, resulting in a non-productive conformation incompetent to bind DNA. Stabilization of the observed quaternary organization of the protein is principally due to the interaction of the linker αL with the lateral face of the EBD (Albanesi et al., 2013). Concerning ligand binding, the structure showed that a tunnel is formed at the interface between the two protomers in the *Sa*FapR homodimer into which the phosphopantetheine group is bound, adopting the same conformation as observed in the truncated *Bs*FapR-malonyl-CoA complex structure, as well as in a number of acyl-CoA-binding proteins harboring the "hot-dog" fold (Albanesi et al., 2013). In this way, the ligand malonate is completely occluded from the bulk solvent. The charged carboxylate group of malonate is neutralized at the bottom of the binding pocket by a specific interaction with an arginine residue. Upon engagement of this arginine in effector binding, a local reorganization is triggered that ultimately leads to surface reshaping and stabilization of the non-productive conformation, thus preventing DNA binding (Albanesi et al., 2013). On the other hand, the adenosine-3′-phosphate moiety of malonyl-CoA is largely exposed to the solvent making no specific contacts with the protein. This implies that *Sa*FapR specifically recognizes the malonyl-phosphopantetheine moiety of the ligand (Albanesi et al., 2013) in agreement with the fact that either malonyl-CoA or malonyl-acyl carrier protein (malonyl-ACP) can both function as effector molecules (Martinez et al., 2010). A detailed comparison of the complexes of full-length *Sa*FapR and the truncated form of *Bs*FapR (lacking the DBDs) with malonyl-CoA revealed a conserved structural arrangement of the EBD core and ligand binding effects. Altogether, the structural alignment indicates an identical mode of malonyl-CoA binding and also the conservation of the DBD–αL–EBD interactions required to stabilize the FapR-malonyl-CoA complex as observed in the *Sa*FapR model (**Figure 2B**) (Albanesi et al., 2013).

The Structure of Full-Length *Sa*FapR

Full-length *Sa*FapR was also crystallized in the absence of ligands (apo-*Sa*FapR) and two crystals forms were obtained (Albanesi et al., 2013). In the different structures, most of the crystallographic independent repressor protomers exhibited the non-productive quaternary arrangement with helix αL bound to the lateral face of the EBD, as observed in the structure of *Sa*FapR in complex with malonyl-CoA (**Figure 2B**), strongly suggesting that in solution the apo-protein would also display this conformation (Albanesi et al., 2013). However, in one of the crystal forms, the helix αL and the associated DBD of one *Sa*FapR protomer could not be modeled due to their high flexibility and

the corresponding first visible residues connecting the helix αL with the EBD exhibited a similar conformation to that found for one subunit of the repressor in the asymmetric *Sa*FapR-DNA complex (**Figure 2A**) (Albanesi et al., 2013). These facts and other crystal parameters (like the extensive crystal contact engagement, the high temperature factors or even the partial disorder displayed by the helix-turn-helix motifs) suggested that alternative conformational states of *Sa*FapR, marked by flexible DBDs, would coexist in solution (Albanesi et al., 2013).

Structural Transitions along the FapR Regulation Cycle

The structural snapshots of full-length *Sa*FapR along its regulation cycle revealed distinct quaternary arrangements for the DNA-bound (relaxed) and the malonyl-CoA-bound (tense) forms of the repressor, with the linker αL involved in different protein-protein interactions in each case, highlighting a functional switch entailing a large-scale structural rearrangement (**Figure 2C**) (Albanesi et al., 2013). Indeed, the amphipathic αL, that in the tense state binds through its hydrophobic face to the protein EBD (**Figure 2B**), dissociates and moves ∼30 Å to finally interact with αL from the second protomer (αL′) and with DNA in the relaxed state (**Figure 2A**) (Albanesi et al., 2013). Furthermore, the structural analysis of apo-*Sa*FapR in two distinct crystal forms also showed that the ligand-free repressor species can populate both, the tense and relaxed conformational states (Albanesi et al., 2013). This suggested that DNA would promote and stabilize the relaxed form of the repressor while an increment in the intracellular concentration of malonyl-CoA would not only trigger the structural changes leading to disruption of the repressor-operator complex but would also drive a shift of the ligand-free *Sa*FapR population toward the tense form (Albanesi et al., 2013).

THE FapR SYSTEM AS A TARGET FOR NEW ANTIBACTERIAL DRUGS

As discussed above, bacterial fatty acid biosynthesis is essential for the formation of biological membranes. Indeed, the importance of the pathway in bacterial physiology is highlighted by the existence of multiple natural products that target different points in this biosynthetic route (Parsons and Rock, 2011). The emergence of resistance to most clinically deployed antibiotic has stimulated considerable interest in finding new therapeutics, leading to a significant effort in academia and industry to develop antibiotic that target individual proteins in fatty acid biosynthesis. One concern about such drugs is that fatty acids are abundant in the mammalian host, raising the possibility that fatty acid synthesis inhibitors would be bypassed *in vivo* (Brinster et al., 2009). Although all bacteria studied to date are capable of incorporating extracellular fatty acids into their membranes, recent research shows that, opposite to what happens in *Streptococcus pneumoniae* (Parsons et al., 2011), exogenous fatty acids cannot circumvent the inhibition of FASII in *S. aureus* and many major human pathogens (Yao and Rock, 2015).

Notably, disruption of FapR-malonyl-CoA interactions by structure-based amino acid substitutions in *S. aureus* leads to permanent repression of fatty acid and phospholipid synthesis, which is lethal and cannot be overcome by addition of exogenous fatty acids (Albanesi et al., 2013), as observed with antibiotics targeting FASII (Parsons et al., 2011). Thus, the distinctive mode of action of FapR together with the promising *in vivo* results highlight lipid homeostasis and the FapR system as a propitious target for the development of new drugs against Gram-positive bacteria.

THE FapR SYSTEM AS A BIOTECHNOLOGICAL TOOL

In the last few years, a number of research groups have taken advantage of the unique properties of FapR to design and construct malonyl-CoA biosensors. Recently, a FapR-based malonyl-CoA sensor has been developed to detect changes of malonyl-CoA flux in living mammalian cells (Ellis and Wolfgang, 2012). After codon optimization, FapR from *B. subtilis* was fused to VP16, a viral transcriptional activator. The VP16 fusion converted FapR from a bacterial transcriptional repressor into a transcriptional activator in the absence of malony-CoA. The FapR operator sequence (*fapO*) was then multimerized and cloned upstream of a minimal promoter driving a reporter gene. This FapR-based malonyl-CoA biosensor was proven to be transcriptionally regulated by malonyl-CoA in mammalian cells and the reporter gene activity was demonstrated to be correlated with the intracellular levels of this effector molecule (Ellis and Wolfgang, 2012). This biosensor was then used to identify several novel kinases that when expressed in COS1 cells (a fibroblast-like cell line derived from monkey kidney tissue) promoted an increment of malonyl-CoA concentrations. In particular, it was shown that the expression of one of these kinases, LIMK1, altered both fatty acid synthesis and fatty acid oxidation rates. Thus, this simple malonyl-CoA responsive biosensor proved to be useful for the study of lipid metabolism in live mammalian cells and the identification of a novel metabolic regulator (Ellis and Wolfgang, 2012).

Two independent groups reported the development of a malonyl-CoA biosensor based on the FapR system of *B. subtilis* in the yeast *Saccharomyces cerevisiae* (Li et al., 2015; David et al., 2016). In both cases FapR was directed to the nucleus where it acted as a repressor on a synthetic promoter containing the FapR-operator site in optimized positions. The biosensors were validated and showed to reflect the change of intracellular malonyl-CoA concentrations. Both groups then used the malonyl-CoA biosensor to improve the production of the biotechnological valuable intermediate 3-hydroxypropionic acid (3-HP), which serves as the precursor to a series of chemicals, such as acrylates. Each group followed a different strategy to achieve this goal. Li et al. (2015) used the malonyl-CoA biosensor to screen a genome-wide overexpression library resulting in the identification of two novel gene targets that raised the intracellular malonyl-CoA concentration. Furthermore, they overexpressed the identified genes in a yeast

strain carrying a bifunctional enzyme, *ca*MCR, from *Chloroflexus aurantiacus* that acts both, as an NADPH-dependent malonyl-CoA reductase and as a 3-hydroxypropionate dehydrogenase, converting malonyl-CoA to malonic-semialdehyde first and then to 3-HP. Interestingly, the authors found that the recombinant yeast strains producing higher amounts of malonyl-CoA showed over 100% improvement of 3-HP production (Li et al., 2015). Using a different approach, David et al. (2016) expressed the gene coding for *ca*MCR (*mcrCa*) under the control of the FapR-based biosensor. This self-regulated system gradually expressed the *mcrCa* gene depending on the available concentration of malonyl-CoA. Subsequently, in order to increase the malonyl-CoA supply for 3-HP production, the authors (David et al., 2016) implemented a hierarchical dynamic control system using the PHXT1 promoter to render FAS1 expression dependent on the concentration of glucose. FAS1 codes for the β-subunit of the fatty acid synthase complex in *S. cerevisiae*, while the α-subunit is encoded by FAS2. The expression of FAS1 and FAS2 is co-regulated, implying a coordinated up—or downregulation of the entire FAS system. Hence, when the external glucose concentration is low the PHXT1 promoter is repressed and FAS1 gene expression is downregulated, decreasing the consumption of malonyl-CoA in fatty acid biosynthesis. As a consequence, there is an increment in the intracellular malonyl-CoA concentration available for 3-HP production. Using this hierarchical two-level control and the fine-tuning of *mcrCa* gene expression, a 10-fold increase in 3-HP production was obtained (David et al., 2016).

Aliphatic hydrocarbons produced by microorganisms constitute a valuable source of renewable fuel so, in order to satisfy the global energy demand, high productivity and yields become essential parameters to achieve. Nowadays big efforts in microbial biofuel production are dedicated to build efficient metabolic pathways for the production of a variety of fatty acid-based fuels. In this regard, two studies were reported on the implementation of the FapR system in *E. coli*, which originally lacks the *fap* regulon, for the improvement of fatty acid production (Xu et al., 2014a; Liu et al., 2015). Malonyl-CoA, produced by ACC (**Figure 1**), is the rate limiting precursor for the synthesis of fatty acids. The *E. coli* ACC is composed of four subunits: a biotin carboxyl carrier protein, a biotin carboxylase, and two carboxyltransferase subunits. The overexpression of the genes coding for the ACC subunits improves fatty acids production but at the same time is toxic to the cells (Davis et al., 2000; Zha et al., 2009). To overcome this drawback, Liu et al. designed a strategy for increasing malonyl-CoA synthesis reducing the toxicity provoked by the concomitant *acc* overexpression (Liu et al., 2015). To this end, they built a negative regulatory system for the *acc* genes based on the ability of FapR to respond to the level of malonyl-CoA. Their goal was to promote a reduction in *acc* expression when malonyl-CoA levels were high and induce it when the malonyl-CoA levels were low. This required the design of a rewired system to create a negative feedback circuit. To this end, the *B. subtilis fapR* gene was cloned into *E. coli* using a low copy number plasmid under the control of a P_{BAD} promoter responding to arabinose. A FapR-regulated synthetic promoter (PFR1) was also constructed by inserting the 17-bp FapR operator sequence into two regions flanking the−10 region of a phage PA1 promoter. PFR1 was validated as a FapR-regulated promoter by analyzing the expression of a fluorescent protein under its control in response to different concentrations of malonyl-CoA (Liu et al., 2015). To complete the circuit, the *acc* genes were placed under the control of a LacI-repressive T7 promoter, PT7, and the *lacI* gene was placed under the control of PFR1. Hence, *acc* expression is initiated upon IPTG induction, producing malonyl-CoA. When malonyl-CoA is accumulated in this strain, the expression from PFR1 will turn on producing LacI, which in turn down-regulates *acc*, decreasing the malonyl-CoA synthesis rate. Using this approach, it was demonstrated that the negative feed-back circuit alleviated growth inhibition caused by either ACC overexpression or malonyl-CoA accumulation (Liu et al., 2015). In addition, this method was used for improving fatty acid titers and productivity and, in principle, could be extended to the production of other chemicals that use malonyl-CoA as precursor (Liu et al., 2015). Xu et al. (2014b) also constructed a malonyl-CoA sensing device by incorporating *fapO* into a hybrid T7 promoter that was shown to be able to respond to a broad range of intracellular malonyl-CoA concentrations, inducing the expression from the T7 promoter at increasing concentrations of the effector molecule. Interestingly, this group then discovered that the FapR protein could activate gene expression from the native *E. coli* promoter P_{GAP} in the absence of malonyl-CoA, that malonyl-CoA inhibits this activation, and that the dynamic range (in response to malonyl-CoA) can be tuned by incorporating *fapO* sites within the P_{GAP} promoter (Xu et al., 2014a). In order to improve fatty acid production, the genes coding for the ACC were then put under the control of the P_{GAP} promoter and the fatty acid synthase (*fabADGI* genes) and the soluble thioesterase *tesA′* were placed under the control of the T7-based malonyl-CoA sensor promoter. Upon constitutive FapR expression, the resulting genetic circuit provided dynamic pathway control that improved fatty acid production relative to the "uncontrolled" strains (Xu et al., 2014a). Taken together, these studies highlight FapR as a powerful responsive regulator for optimization and efficient production of malonyl-CoA-derived compounds.

CONCLUSIONS AND PERSPECTIVES

FapR is a global transcriptional repressor of lipid synthesis highly conserved in Gram-positive bacteria. Notably, the activity of this repressor is controlled by malonyl-CoA, the product of the first dedicated step of fatty acid biosynthesis, converting FapR into a paradigm of a feed-forward-modulated regulator of lipid metabolism. The activity of other well-characterized bacterial lipid regulators, like FadR of *E. coli* (van Aalten et al., 2001) or the TetR-like *P. aeruginosa* DesT (Miller et al., 2010), is feedback controlled by the long-acyl chain-end products of the FASII pathway (Zhang and Rock, 2009; Parsons and Rock, 2013). The EBDs of these proteins, frequently exhibit an α-helical structure with a relaxed specificity for long-chain acyl-CoA molecules, possibly because helix-helix interactions are permissive enough to constitute a platform for the evolution of a binding site for fatty acids of diverse chain lengths (Albanesi et al., 2013). In

contrast, the feed-forward regulation mechanism of the FapR repressor family, which implies the recognition of the upstream biosynthetic intermediate malonyl-CoA, requires a high effector-binding specificity. In FapR, this high specificity is achieved by confining the charged malonyl group into a quite rigid internal binding pocket, and may be the reason why the "hot-dog" fold was recruited for this function (Albanesi et al., 2013). It is important to note that organisms using the FapR pathway could also count on a complementary feed-back regulatory loop operating at a biochemical level, for instance by controlling the synthesis of malonyl-CoA (Paoletti et al., 2007). If this is proven, it would imply that FapR-containing bacteria finely tune lipid homeostasis by feed-back and feed-forward mechanisms, as it indeed happens in higher organisms ranging from the nematode *Caenorhabditis elegans* to humans (Raghow et al., 2008).

Human health and life quality have significantly improved with the discovery of antibiotics for the treatment of infectious bacterial diseases. However, the emergence of bacterial resistance to all antimicrobials in clinical use (Levy and Marshall, 2004; Davies and Davies, 2010) has caused infectious bacterial diseases to re-emerge as a serious threat to human health. This scenario highlights the need to develop new strategies to combat bacterial pathogens. FapR controls the expression of many essential genes for bacteria not only involved in fatty acids but also in phospholipid synthesis. It has been experimentally shown that the presence of mutant variants of FapR unable to bind malonyl-CoA result lethal for bacteria (even in the presence of exogenous fatty acids), as the regulator remains permanently bound to DNA impeding the expression of its target genes. These results and the existence of FapR in important human pathogens validate FapR and lipid homeostasis as interesting targets for the search of new antibacterial drugs. With another perspective, the high specificity of FapR for malonyl-CoA has allowed for the development of *in vivo* malonyl-CoA sensors in diverse organisms that originally lack FapR and the *fap* regulon. These sensors have been shown to function in mammalian cells, in yeast and in bacteria responding accurately to the intracellular variations in the concentration of malonyl-CoA. The different FapR-based-malonyl-CoA biosensors were constructed following alternative strategies and used with a broad range of purposes focused on biological processes involving malonyl-CoA, including signaling mechanisms and metabolic engineering. Malonyl-CoA is the precursor of many industrial-valuable compounds like fatty acids, 3-hydroxypropionic acid, polyketides, and flavonoids, since they can be used as or converted to biofuels, commodity chemicals, fine chemicals, and drugs. Due to the success in the implementation of the FapR-based biosensors to improve the productivity and yields of the production of several malonyl-CoA-derived compounds, it is expected that new biotechnological applications of the FapR system emerge in the short term.

AUTHOR CONTRIBUTIONS

DA and DdM conceived and wrote this review.

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Review

Control of membrane lipid homeostasis by lipid-bilayer associated sensors: A mechanism conserved from bacteria to humans

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ABSTRACT

The lipid composition of biological membranes is key for cell viability. Nevertheless, and despite their central role in cell function, our understanding of membrane physiology continues to lag behind most other aspects of cell biology. The maintenance of membrane properties in situations of environmental stress requires homeostatic sense-and-response mechanisms. For example, the balance between esterified saturated (SFAs) and unsaturated fatty acids (UFAs), is a key factor determining lipid packing, water permeability, and membrane fluidity. The reduced thermal motion of lipid acyl chains triggered by an increase in SFAs causes a tighter lipid packing and increase the membrane viscosity. Conversely almost all organisms adapt to membrane rigidifying conditions, such as low temperature in poikilotherms, by incorporating more lipids with poorly packing unsaturated acyl chains. The molecular mechanisms underlying membrane homeostasis are only starting to emerge through combinations of genetics, cell biology, lipidomics, structural approaches and computational modelling. In this review we discuss recent advances in defining molecular machineries responsible for sensing membrane properties and mediating homeostatic responses in bacteria, yeast and animals. Although these organisms use remarkably distinct sensing mechanisms to mediate membrane adaptation, they suggest that the principle of transmembrane signaling to integrate membrane composition with lipid biosynthesis is ancient and essential for life.

1. Introduction to biological membrane lipid composition

Lipid bilayer membranes have been a defining feature of cells since their very origin several billion years ago [1]. Today we find numerous instances of membrane composition adaptation to variations in environmental conditions such as temperature, pH or pressure across all of life's diversity [2–4]. Bacterial cytoplasmic membranes, composed of roughly equal proportions of proteins and lipids, have a multitude of important essential functions. The membrane comprises the major boundary outlining the cell cytoplasm, and it controls the transport and diffusion of myriads of small molecules and secreted proteins between the intracellular and extracellular space. The transmembrane electrochemical gradient powers many crucial cell functions. Different lipid components are assumed to contribute to specialization of these crucial functions, but we know little about the molecular details of these effects. Further, the specific composition of subcellular membranes in eukaryotes is of paramount importance for many cellular processes ranging from vesicular trafficking to organelle homeostasis, mitochondrial respiration and receptor signaling [5–9]. It is therefore not surprising that many disease states are associated with membrane composition defects. Diabetics, for example, have rigid cellular membranes that likely contributes to many aspects of the pathophysiology of this disease such as poor microcirculation and impaired insulin signaling [10–12]. Similarly, defects in membrane composition are hallmarks of cancer cells [13,14] and also likely contribute to protein aggregation in Parkinson's and other neurodegenerative diseases [15–17]. "Membrane-lipid therapy" is therefore a promising new front in the treatment of several diseases [18].

Given its central and far-reaching importance, it is surprising that so little is known about the molecular mechanisms that regulate membrane composition. However, this is now changing. New or improved methods, such as lipidomic analysis of membrane composition [19–23], fluorescence recovery after photobleaching (FRAP) and other methods to measure membrane fluidity in vivo [24–26], molecular modelling of membranes [27–29] and powerful genetic approaches in bacteria, yeast and animals have recently led to the identification of regulators of membrane homeostasis, and elucidation of their mechanism of action. We now understand that the homeostasis of cellular membranes is a wonderful example of self-organization. It relies on gradients of membrane composition generated by synthesis of new lipids in the ER, where membrane disorder is highest and therefore most permissive, then increasingly specialized membrane composition through the secretory pathways, with the plasma membrane being the most rigid, impermeable and selective membrane. The composition and properties of the distinct subcellular membranes, which are adapted to function, is monitored and guarded by several sense-and-response proteins. These

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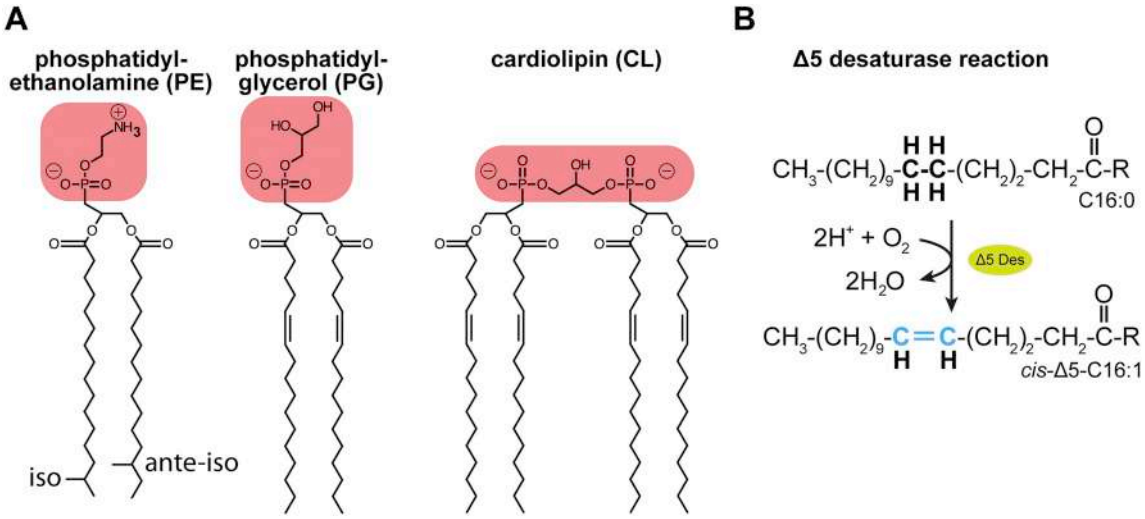


Fig. 1. Examples of bacterial phospholipids and Δ5-desaturase activity. (A) Illustration of the three most common phospholipids in *E. coli*, with the hydrophilic head groups highlighted in red. Note the *iso*- and *anteiso*-branched fatty acids in the PE and the monounsaturated fatty acids in the PG and CL examples. (B) Desaturation of fatty acids mediated by the Δ5-Des acyl lipid desaturase. The R group is the 1-acyl lysophospholipid moiety of membrane phospholipids. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

self-organizing principles and sense-and-response sensors are the subject of this review. We will begin by introducing the importance of fatty acid desaturation during bacterial adaptation to growth at low temperature, with an in-depth description of the best understood membrane sense-and-response protein, namely DesK. The second part of the review will focus on membrane composition and homeostasis in eukaryotes.

2. Membrane homeostasis in bacteria

2.1. Temperature regulation of fatty acid composition in bacteria

The bacterial cytoplasmic membrane is built upon the familiar lamellar phospholipid bilayer structure, an extended two dimensional surface of two opposed monolayers [30]. Although there is a considerable diversity of phospholipid structures in the bacterial world, most membrane phospholipids are glycerolipids that contain two fatty acid chains [30]. The cytoplasmic membrane of *E. coli*, for example, is composed primary of the phosphatidylethanolamine (75%), phosphatidylglycerol (20%) and cardiolipin, which is composed of two phosphatidic acids connected via a glycerol group (Fig. 1A) [31]. The lipid bilayer of the cytoplasmic membrane forms a hydrophobic barrier that prevents the uncontrolled movement of polar molecules and allows the accumulation and retention of metabolites and proteins [32]. The lipids also provide a suitable environment for the proper functioning of the membrane protein complexes involved in bioenergetics and biosynthetic functions. The lipid composition determines the dynamics and interactions of lipid molecules, which in turn determine the barrier and permeability properties of the membrane and influence the topology, interactions and functions of the membrane proteins [33,34]. The functions of the cytoplasmic membrane are known to depend critically on the physical state of the lipid bilayers, making it susceptible to changes in environmental temperature [35–37]. Environmental temperature is therefore a critical variable for microorganisms because it equilibrates quickly with their small volumes, modulating steeply the reaction rates and equilibria that underlie cellular biochemistry. In particular, it has been established that normal cell function requires membrane lipid bilayers that are largely fluid; indeed, the bilayers of most organisms are entirely or mostly fluid at physiological temperatures [30,38]. However, at low temperature membrane bilayers undergo a reversible change of state from a fluid (disordered) to a nonfluid (ordered) array of the fatty acyl chains [38,39]. The temperature at the midpoint of this transition is called the transition temperature (T_m), and the change of state accompanying an increase in temperature is called the liquid-gel transition [38]. The T_m is a function of the membrane lipid composition and, in organisms deficient in cholesterol, mainly depends on the fatty acid composition of the membrane lipids [38,39]. A disordered state is imparted by the presence of either unsaturated or terminally branched fatty acids both of which act to offset the closely packed ordered arrangement of the lipid bilayer acyl chains that is imparted by straight chain-saturated acyl chains [30]. Straight-chain saturated fatty acids, such as hexadecanoic acid (C16:0), are linear and pack together efficiently to produce a bilayer that has a high T_m and low permeability properties. The *cis* unsaturated fatty acids (UFAs) introduce a pronounced kink in the chain, which disrupts the order of the bilayer and results in lower transition temperature and higher permeability. The composition of the branched-chain fatty acids affects membrane fluidity owing to the disruptive effect of the methyl group on acyl chain packing. The *anteiso*-branched fatty acids (a-BCFA) promote a more fluid membrane than the *iso* fatty acids, because the methyl branch is further away from the end of the fatty acid [30,40,41].

From these considerations, it seems clear that bacteria and most (if not all) organisms unable to maintain thermal homeostasis must regulate their plasmatic membrane phase transition in response to temperature [42]. Without regulation, an organism shifted from a high to a low temperature would have membrane lipids with suboptimal fluidity, resulting in subnormal membrane function, and an organism shifted from low to high temperature would be too fluid and potentially leaky. This membrane lipid homeostasis that maintains the biophysical properties of membranes is referred to as homeoviscous adaptation [43], and is interpreted as a mechanism that modifies the permeability of the phospholipid bilayer to minimize energy expenditure and optimize growth [30].

The mechanism of homeoviscous adaptation in all cases examined seems to occur via the incorporation of proportionally more UFAs (or fatty acids of analogous properties, such as *a*-BCFAs) as the temperature decreases [44,45]. This regulatory mechanism system seems to be a universally conserved adaptation response allowing cells to maintain the appropriate fluidity of membrane lipids regardless of the ambient temperature [46]. This means that cells must process temperature signals to adjust enzyme activities or to activate unique genes necessary to adapt the membranes to the new temperature [42]. While the activities of all the biomolecules are altered as a function of temperature, the thermosensors we focus on here are proteins that sense changes in the

lipid bilayer environment triggered by a sudden decrease in temperature. These sensors mediate the cold-dependent transcriptional induction of acyl lipid desaturase enzymes that introduce *cis*-double bonds into preexisting fatty acids, thus optimizing membrane fluidity at the new temperature. Here, we initially provide an overview of the work on bacterial acyl lipid desaturases in Bacillus. Then, the focus is set mainly on the recent biochemical and structural insights into the paradigmatic DesK thermosensor of the model soil bacterium *Bacillus subtilis*. The *B. subtilis* centric tone of these review sections reflects the fact that most of the work on control of membrane fluidity by a molecular thermometer has been carried out on this model Gram-positive bacterium. Later sections will examine membrane property sensors in eukaryotes.

2.2. Unsaturated fatty acid biosynthesis

Konrad Bloch, a Nobel Laureate chemist, and coworkers determined that UFAs are synthesized in two different ways; one occurs only in aerobic organisms and requires molecular oxygen, while the other pathway is used in anaerobic conditions [47,48]. The anaerobic pathway of UFAs biosynthesis was extensively studied in the *Escherichia coli* model system and has been comprehensively reviewed. Here, we will briefly describe the aerobic pathway of UFAs synthesis which functions in all eukaryotic forms of life and certain bacteria [40,45] (Fig. 1B). In this pathway the double bond is introduced post-biosynthetically into saturated fatty acids by desaturase enzymes [49]. Desaturase enzymes perform dehydrogenation reactions that result in the introduction of double bonds into fatty acids through a mechanism initiated by the energy-demanding abstraction of a hydrogen atom from a methylene group [49]. To achieve this, desaturase enzymes recruit and activate molecular oxygen with the use of a diiron cluster located in their active sites [49]. The diiron center is common to a variety of proteins, including methane monooxygenase, ribonucleotide reductase, rubrerythrins, and a variety of oxidase enzymes [49]. Desaturase enzymes have evolved independently twice; the acyl-carrier protein desaturases are soluble enzymes found in the plastids of higher plants [50], whereas the more widespread class of integral membrane desaturases is found in endomembrane systems in prokaryotes [45,51] and eukaryotes [52,53]. The membrane-bound desaturases, which introduce the double bond in fatty acids sterified to glycerolipids, are named acyl-lipid desaturases [51]. All these enzymes utilize reducing equivalents obtained from an electron transport chain and are able to introduce the double bond in a chemo-, regio-, and stereoselective manner [49]. Three main types of specificities for the introduction of the double bond (regioselectivity) have been observed for fatty acid desaturases that would reflect differences in the position of the active site relative to the features of the substrate binding pocket associated with substrate recognition: the Δx desaturases introduce a double bond x carbons from the carboxyl end; ω-x desaturases dehydrogenate x carbons from the methyl terminus; while $\nu+x$ desaturases use a preexisting double bond as a reference point and dehydrogenate x carbons from the nearest olefinic carbon [54].

2.3. Transcriptional regulation of lipid desaturation in Bacillus species

Konrad Bloch and coworkers were the first to describe the existence of an oxidative pathway for the biosynthesis of long-chain UFAs by microorganisms, thus initiating the study of bacterial desaturases [55]. Fulco and Bloch demonstrated desaturation in several bacteria, including *Bacillus megaterium* [56–58]. One of the most significant discoveries performed by these researchers, at least to understand thermosensing via changes in membrane properties, was the remarkable discovery of temperature dependence of the desaturation reaction in *B. megaterium* [56](for a historical perspective see reference [59]). Thus, at a growth temperature of 23 °C, radioactive palmitate added to the medium was almost completely desaturated to an UFA containing a *cis*-double bond at position 5 relative to the carboxyl end of the fatty acid (Δ5), while at 30 °C desaturation was negligible [56]. Presumably, this inverse relationship between temperature and desaturation functioned in the regulation of membrane fluidity in response to fluctuations in growth temperature but the mechanism was totally unknown. After this initial discovery, Fulco and coworkers went on with the characterization of this adaptive response demonstrating that the fatty acid desaturation system was induced when cultures of *B. megaterium* were grown at low temperatures [60,61] (for reviews see [62,63]). It was also found that the levels of desaturation of cultures of *B. megaterium* transferred from 35 to 20 °C far exceeded the levels of desaturation of cultures growing at 20 °C [64]. To explain the dramatic change in the lipid composition of bacilli shifted from 35 to 20 °C, it was proposed that transcription of the fatty acid desaturase gene occurs only at low growth temperatures. To account for the initial degree of desaturation seen immediately after a downward temperature shift, Fujii and Fulco [65] postulated the existence of a modulator protein whose synthesis also proceeds at lower temperatures but only following a brief delay. Thus, the rapid desaturation taking place in freshly downshifted cells would soon be moderated to a rate yielding the steady-state level of fatty acid unsaturation characteristic at that temperature. However, no direct experimental evidence supported this proposed “on-or-off” transcriptional regulatory model of desaturase synthesis. To further explore the molecular mechanism of cold induction of UFA biosynthesis and how a change in growth temperature regulates the expression of the *Bacillus* desaturase, de Mendoza and his co-workers decided to study this phenomenon in *B. subtilis*, which is an excellent experimental model because of its general experimental tractability. Like *B. megaterium*, *B. subtilis* growing in rich medium at 37 °C almost exclusively synthesizes saturated fatty acids [66]. However, when a culture grown at 37 °C is transferred to 20 °C, the synthesis of UFAs is induced. As with *B. megaterium*, the desaturation system of *B. subtilis* requires de novo synthesis of RNA and proteins, since it is completely abolished by rifampin or chloramphenicol added before a downward temperature shift [66]. The *des* gene, encoding the sole desaturase of *B. subtilis*, was isolated by complementation of *E. coli* strains with mutations in either the *fabA* or *fabB* genes, which are essential for UFA synthesis [67]. The *des* gene encodes a polytopic membrane-bound desaturase which requires molecular oxygen as an electron acceptor [68] and catalyzes the introduction of a *cis* double bond at the Δ5 position of a wide range of saturated fatty acids [69], using either Ferredoxin or Flavodoxin [70] as electron donors. This protein was named Δ5-Des [69]. A detailed transcriptional analysis demonstrated that the *des* gene is tightly regulated during cold shock [71]. The induction of *des* mRNA takes place in the absence of new protein synthesis, indicating that the desaturase transcript can be produced upon cold shock by using already existing resources at the time of the temperature downshift [71]. In addition, the level of the *des* transcript produced in a *B. subtilis* strain in which the wild-type *des* promoter was exchanged with the *spac* promoter was not decreased after continuous growth at 20 °C [71]. This finding indicates that the transient induction of the wild-type *des* gene at low growth temperatures was due to the promotion of transcription rather than to the stabilization of the *des* mRNA. This would explain why, similar *to B. megaterium*, the level of UFAs synthesized by *B. subtilis* during the first growth division cycle was much higher than those of cultures growing for several generations at 20 °C [66].

2.4. DesK-mediated temperature sensing

DesK was discovered around 15 years ago as the sensor protein of a two-component system that maintains appropriate membrane fluidity at low growth temperatures in *B. subtilis* [72]. DesK is encoded by the first gene of a small operon coding also for its cognate response regulator, DesR, and its only target is the *des* gene, which codes for Δ5-Des [67,71]. Transcription from the P*des* promoter is triggered in *B. subtillis* when the temperature drops below ~30 °C, inducing desaturase expression. Desaturase activity then introduces *cis*-double bonds into acyl

chains that are attached to existing phospholipids, helping to restore appropriate membrane fluidity in the new environment [67,71]. Below we summarize our current understanding and hypotheses about DesK functioning. We analyze the structural features of DesK in the two main functional states of the effector domain, discuss current proposals on how the primary cold signal is sensed by the TM region and transmitted to the effector domain through the TM and 2-HCC regions, and advance possible roles for unexplored conserved prolines of the TM domain.

2.5. General architecture of DesK

DesK is a transmembrane histidine kinase (HK) lacking extracellular domains. When active as a kinase, DesK phosphorylates the response regulator DesR, which activates the transcription function of DesR for the *des* gene. The transmembrane (TM) region of DesK is composed of 10 TM helices, 5 coming from each protomer (TM1-5), arranged in an unknown fashion. TM5 connects directly into a signaling helix that ends up in the Dimerization and Histidine phosphotransfer (DHp), a four helix bundle (4-HB) domain, followed by a catalytic and ATP binding domain. Systematic deletion of DesK's TM segments showed that the TM topology can be simplified, retaining functionality. More precisely, a truncated minimal sensor version in which the first half of the first TM helix is fused to the second half of the fifth TM helix, dubbed MS-DesK, is fully functional both *in vitro* and *in vivo* [73] (Fig. 2A, B). Full-length DesK and MS-DesK have been the subject of several structural and functional studies, shedding light on the mechanism underlying temperature sensing and putting forward some general ideas about HK functioning.

The first structural study on DesK reported nearly complete x-ray structures for its cytoplasmic portion (DesKC) in different functional states, including phosphatase and kinase-competent states [74]. These structures highlighted a conformational change at the DHp and the 2-helix coiled coil (2-HCC) N-terminal to the DHp, central to the mechanism of activation into the kinase-competent form (Fig. 2A, B).

Close comparison of DesKC structures hints at rotation and tilting of the two helices that enter the DHp from its N-terminal side (each helix coming from one protomer) as a route for signal transduction into DesKC (Fig. 2C–H). More precisely, each helix appears rotated by roughly 90° around its long axis on its N-terminal end where the construct begins, a few residues after TM5. These rotations are such that the helices establish hydrophobic contacts in the phosphatase-competent state but not in the kinase-competent state, where some hydrophobic residues of the 2-HCC and the DHp 4HB become exposed, *i.e.* a destabilized conformation (Fig. 2C–H).

2.6. Temperature sensing relies on membrane status as a proxy

The genetic and biochemical studies that led to the discovery of the Des pathway in *B. subtilis* suggested that DesK could assume different signaling states under varying growth temperatures [72]. Later, a set of *in vivo* and *in vitro* studies on full-length DesK and truncated constructs in the TM domain suggested that DesK detects membrane thickness as a cue for cold sensing [73,75]. By increasing truncation of DesK's TM helices [73], de Mendoza's group observed that TM1 and TM5 are both required for temperature-dependent regulation of the kinase output. They next observed that DesK constructs containing either TM1 or TM5 alone could not respond to the cold signal, whereas the chimeric TM1/5 construct MS-DesK responded to the cold signal similarly to full-length DesK [73]. Using this minimal construct, they further tested the effect of amino acid substitutions and insertions in the transmembrane portion of its helix, concluding that hydration, packing and hydrophobic match to the membrane in MS-DesK's single TM helix tune functional output. In particular, the observation that altering the length of the hydrophobic segment of the TM1/5 chimeric helix alters functional output suggested that it acts as a ruler of membrane thickness [73,75]. Further experiments on MS-DesK with the wild type sequence reconstituted in liposomes of different membrane thicknesses showed a more rapid and overall higher kinase output in liposomes made of lipids with longer acyl chains [73]. Moreover, *in vitro* and *in vivo* experiments with full-length DesK also showed that lipids with longer acyl chains promote activation of the kinase activity of the sensor [75,76]. These results indicated that MS-DesK and DesK respond to membrane thickness by modulating its catalytic output. This however does not prove that thickness itself is the true signal *in vivo*, as the increment in thickness of *B. subtilis* membrane phospholipids is in the order of 1 Å when going down from 37 to 25 °C [77], *i.e.* smaller than the difference in thickness of the proteoliposomes where the activity of MS-DesK [73] and DesK [76] was stimulated *in vitro*. It is important to note that membrane properties other than thickness are affected by temperature and acyl chain lengths, including permeability to water and fluidity/rigidity, especially when fluid-to-gel phase transitions are caught in the relevant temperature range, hampering a clear assessment of which properties are actually sensed by the protein [30,78–80]. In any case, it is clear that DesK senses membrane status as a proxy for cold via mechanisms rooted in the internal mechanics of the TM bundle.

2.7. A possible sensing mechanism for DesK

Comparison of DesKC in the phosphatase and kinase conformations (Fig. 2 Lower panel) suggested that the contacts between the DHp and the ATP binding domains (ABDs), as well as the parallel 2-HCC, support a labile association that is released for autophosphorylation and maintained for the phosphatase activity under control of the sensor domain [74]. Therefore, the coiled coil is expected to be stable in the phosphatase competent state but disrupted in the kinase-competent state. The role of 2-HCC stability in DesK function was directly tested by studying mutants aimed at stabilizing and destabilizing it. In the destabilized mutant, hydrophobic residues of the cytosolic portion of the 2-HCC were replaced by hydrophilic residues; in the stabilized mutant, polar residues close to the exit of TM5 from the membrane were replaced by hydrophobic residues [81]. Atomistic models of the wild type and stabilizing/destabilizing mutants, as well as experimental activity measurements clearly proved that the stabilized 2-HCC is associated with the phosphatase-competent conformation whereas loosened coiled coil packing favors the kinase-competent state [81]. Therefore, it was concluded that switching of DesKC's catalytic output is determined by stabilization/destabilization of the 2-HCC, very likely through rotations along their long axes and through separations leading to kinase activation. Linking these experimental findings to other available functional evidence to make further interpretations and speculate about how 2-HCC rearrangements could be triggered and transmitted by the TM region is hampered by the lack of structures or models of full-length DesK. Therefore, atomistics models of MS-DesK were built by extending structures of DesKC in the phosphatase and kinase states to include the full chimeric TM1/TM5. Atomistic molecular dynamics (MD) of these MS-DesKC were built and relaxed in explicit solvent and membranes composed of di(C18:1) PC and dieurocoyl PC [di(22:1)PC], the latter being about 4.5Å thicker. The phosphatase model relaxes in di(C18:1) PC maintaining a symmetric structure and a stable 2-HCC all the way through the membrane and the cytoplasm into the DHp, with distorsions introduced locally at the two prolines of each helix (Pro16 and 148 in DesK numbering) [81,82]. On the contrary, in the thicker membrane of di(22:1)PC the kinase drifts away from its starting conformation adopting stretched parallel TMs [81,82], instead of a coiled arrangement, as observed in two nearly full structures of histidine kinases in their kinase competent forms [83,84].

The genetic, biochemical, structural and computational studies [74,81,82] suggests that membrane inputs related to cold-induced thickening trade off with the protein's internal mechanics, particularly those concerning 2-HCC stability and its helical rotations. Upon a drop in temperature the membrane becomes thicker and more structured, imposing on the 2-HCC a stress that results in its conversion from the

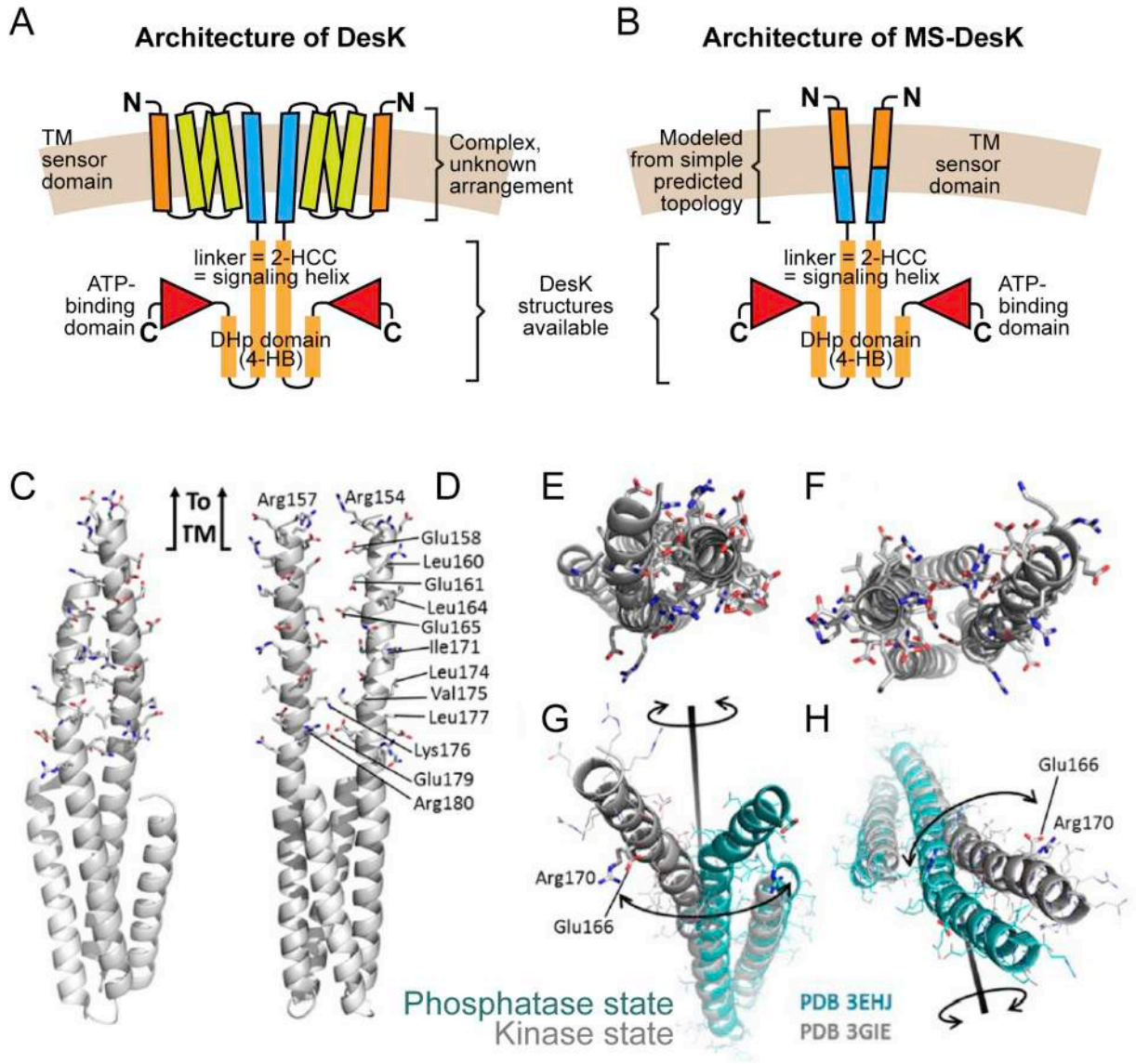


Fig. 2. Architecture of DesK and structural basis of DesK regulation. (A) Architecture of *B. subtilis* DesK with a simple helical linker connecting the TM cold sensing domain with the cytoplasmic domain (DesKC). (B) Architecture of MS-DesKC, a functional minimal version of DesK. Nt = N terminus and Ct = C terminus. DesK's DHp domain and 2-HCC linker in the phosphatase-like state (C, E and G-H PDB ID 3EHJ) or opened apart in a kinase-like state (D and F and G-H PDB ID 3GIE). The backbone trace spans from the first observed residue (154–158 depending on PDB entry and chain) until Tyr210. Side chains of 2-HCC residues (until Arg185) are shown as sticks (red = oxygen, blue = nitrogen, grey = carbon). Some hydrophobic residues that become exposed and charged residues that become buried in the kinase state are labelled in panel D. Panels G and H compare chains A and B from both structures to highlight the 90 degrees rotations of the helices as exemplified through Glu166 and Arg170. Interactive 3D views are available at https://lucianoabriata.altervista.org/papersdata/accounts2017.html. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

relaxed phosphatase state (Fig. 3A) into the autokinase-competent state (Fig. 3B). From this model it seems clear that the main input into DesKC is a 90° rotation and separation of the signalling helices that make up the 2-HCC and connect into the DHP. But what drives helical rotation upon sensing? Unbiased atomistic MD simulations of each of DesK´s TM helices suggest that membrane fluidity increases helix mobility of only TM1 and TM5 in full length DesK [82]. This information suggests that TM1 and TM5 could be the key sensors elements, in line with MS-DesK being functional [73]. The simulations further showed kinking of the TM helices at proline residues, interestingly Pro16 and Pro148, which locate internally (more than 2 helical turns away from the closest helix terminus). Proline itself has interesting structural properties, introducing kinks and flexible hinges in TM helices, playing a key role in signalling proteins. A recent report [85] showed that replacement of proline 148 (in DesK numbering) in MS-DesK inactivates its kinase activity. This observation agrees with simulation studies suggesting that Pro148-induced kink could guide rearrangements of helices 1 and 5 to drive scissoring motions that shifts between compact and loosened cytoplasmic 2-HCC in full length DesK [82]. These exploratory simulations and analysis, although not conclusive, clearly point to prolines as the subject of future research in DesK in transmembrane signalling.

3. Membrane homeostasis in eukaryotes

3.1. General principles

As in bacteria, the primary component of eukaryotic membranes are the phospholipids (Fig. 4A–B), which are composed of a hydrophilic glycerol 3-phosphate-derived head group attached to two fatty acids at positions sn-1 and sn-2 of the glycerol molecule. The fatty acids can vary in length (mostly between 16 and 25 carbons) and degree of unsaturation (examples are shown in Fig. 4C). Fatty acids considered to be

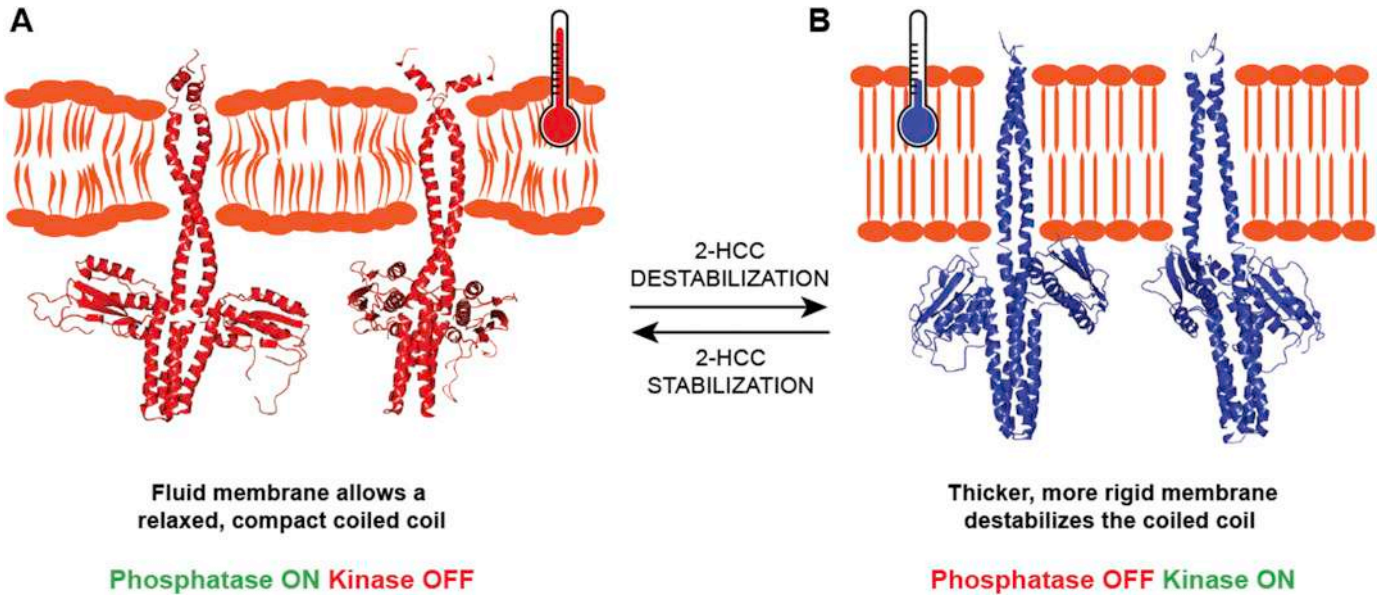


Fig. 3. The coiled coil switch mechanism of temperature sensing and transduction by DesK. (A) High temperatures promote thin and fluid membranes allowing a relaxed structure of DesK, characterized by the formation of a 2-HCC through the entire TM5 segments and its cytoplasmic extensions towards the 4-HB, plus the tight interactions between the DHp and ABD domains, corresponding to the characteristic structure of the phosphatase-competent state. (B) Upon a temperature drop the membrane thickens, stretching the TM segments, inducing the unwinding of the 2-HCC and a gradual rotation of its helices up to approximately 90°, bringing large charged residues to the interior of the 2-HCC and forcing its destabilization. These rotations are transmitted to the 4-HB, which now hides the residues that were generating the extensive interaction surface with the ABDs, inducing their release to reach the auto-kinase conformation.

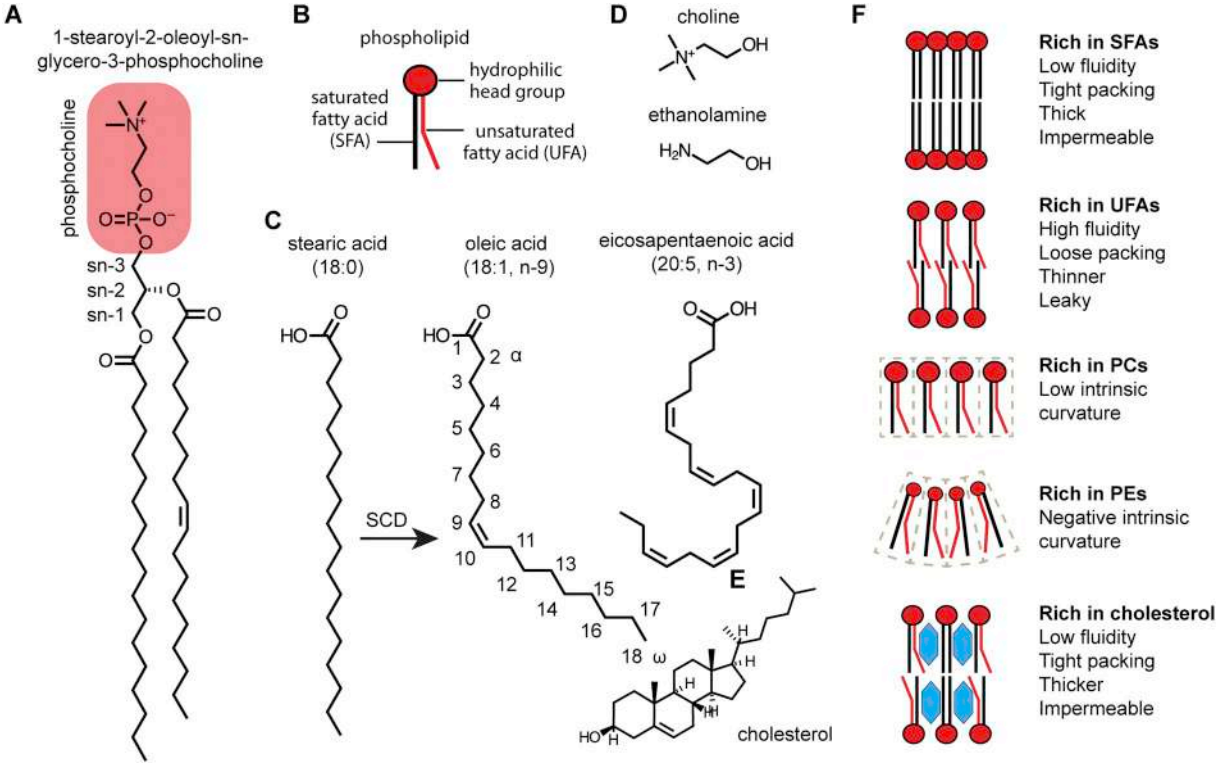


Fig. 4. Components and features of membranes. (A) Structure of a phospholipid, with the hydrophobic head group highlighted in red, and the sn-1, sn-2 and sn-3 positions indicated. Note that one UFA (unsaturated fatty acid) is present at position sn-2, which is typical of phospholipids. (B) Cartoon representation of a phospholipid. (C) Examples of FAs (fatty acids), including one SFA (saturated fatty acid; stearic acid), one MUFA (monounsaturated fatty acid; oleic acid) and one VLCPUFA (very long chain polyunsaturated fatty acid; eicosapentaenoic acid); the activity of a Δ9-desaturase, SCD, is also indicated to convert stearic acid to oleic acid. (D) Examples of two hydrophobic head groups: the relatively large choline and the relatively small ethanolamine, which form cylindrical and conical phospholipids, respectively. (E) Structure of cholesterol. (F) Annotated representation of the effect of membrane composition, i.e. SFA/UFA content and relative abundance of PCs (phosphatidylcholines), PEs (phosphatidylethanolamines) and cholesterol, on membrane properties. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rigidifying are mostly saturated, which facilitates their tight packing. Conversely, the most fluidizing phospholipids are the long-chain polyunsaturated fatty acids (LCPUFAs), such as the omega-6 arachidonic acid (AA; C22:4, n-6), the omega-3 eicosapentaenoic acid (EPA; C20:5, n-3) or the omega-3 docosahexaenoic acid (DHA; C22:6, n-3), which are too long to fit straight in the membrane and are highly kinked by the presence of multiple cis-double bonds. Most naturally occurring phospholipids have an asymmetric FA distribution, with the sn-1 position usually occupied by a SFA and the sn-2 position occupied by a UFA [86–88]. This asymmetry is important to balance permissivity to vesiculation while maintaining a proper control of membrane permeability [89].

The nature of the hydrophilic head group is also important. For example, phosphatidylcholines (PCs) tend to form “cylinder” shaped phospholipids because of the rather large choline head group while phosphatidylethanolamine (PEs) forms “conical” phospholipids because of the smaller ethanolamine head group (Fig. 4D). A consequence of these shape differences is that PCs naturally pack more easily and tend to form flat membranous structures; PCs therefore account for > 50% of the phospholipids in most eukaryotic membranes. On the other hand, PEs generate negative curvature strain that in a bilayer membrane can result in packing gaps due to the small head group and bulky acyl chains (Fig. 4C, D). Modulating the relative abundance of PCs and PEs in membranes is therefore an effective way to influence membrane fluidity [90], and attempts to manipulate the ratio between cylindrical and conical lipids triggers compensatory mechanisms, as documented in yeast (e.g. shortening and increased saturation of the lipid acyl chains to compensate for depletion of PCs) [91]. Sterols, such as cholesterol (Fig. 4E), are also an abundant component of many eukaryotic membranes. Sterols are hydrophobic rigid structures that can increase fluidity when inserted in rigidifying phospholipids rich in saturated fatty acids or decrease fluidity when inserted among fluidizing phospholipids rich in UFAs [92]. Note that in some instances, PEs mimic cholesterol to impart rigidity because the packing pressure in their acyl chains may increase to compensate for the packing gaps at the surface [90]. Some effects of membrane composition are illustrated in Figs. 4F and 5. Note that different fluid phases delimited by phase boundaries can co-exist within the same eukaryotic cell membrane [93–95], and contribute to many of its properties, including providing specialized domains for the recruitment and activity of membrane proteins.

Excellent overviews of the physicochemical properties of biological membranes (fluidity, thickness, permeability, packing, phase behaviour, curvature, response to compression, lateral heterogeneity and charge) have been written [96–99]. For the purpose of this review, the term “fluidity” will be used as a catch-all term for many of these properties, correlating mostly with a higher UFA content within phospholipids.

3.2. Variation in membrane lipid composition

An overview of membrane homeostasis in animal cells is presented in Fig. 6, with emphasis on concepts discussed in the present review. The lipid composition of cellular membranes varies greatly according to their functions. The endoplasmic reticulum (ER) membrane, which must be highly fluid and flexible to accommodate all types of newly synthesized lipids and membrane proteins, which both generally have half-lives of ~100 h in hepatocytes [100], is rich in phospholipids containing short-chain and unsaturated fatty acids, and is poor in cholesterol [101,102]. In contrast, the plasma membrane, which must form an impermeable barrier against the extracellular environment, is much more rigid because it is rich in PCs and cholesterol, and in phospholipids containing long-chain and saturated fatty acids. There is thus a rigidity gradient along the membrane synthesis/secretory pathway ranging from great fluidity and permeability in the ER, through gradually reduced fluidity across the trans-Golgi to the cis-Golgi, and finally great rigidity and impermeability in the plasma membrane [102–104]. This fluidity gradient influences protein localization in the various organelle; for example, proteins with shorter transmembrane domains are easily accommodated in the ER membrane while proteins with longer transmembrane domains will localize to thicker membranes, such as the plasma membrane [105].

Cell types also vary in their membrane composition according to their functions. For example, the brain is rich in LCPUFAs, especially docosahexaenoic acid (DHA; 22:6, n-3) [106], because of the membrane fusion requirements involved in synaptic vesicle neurotransmitter release; DHA therefore accounts for over 10% of total FAs in synaptosomal membranes [107] and is distributed as a concentration gradient along axons grown in vitro with highest levels at the ends of the axons [108]. The environment also influences membrane composition. Consider the fact that almost all DHA is obtained from marine oils produced by phytoplankton then assimilated at all levels of the marine food chain. Ultimately, this phenomenon is driven by the fact that active metabolism in cold water requires the highly fluidizing properties of LCPUFAs to maintain sufficient membrane fluidity. These same omega-3 fatty acids are essential dietary fatty acids in human because we cannot synthesize them; a special transporter exists to insure their efficient uptake by the brain [109]. Conversely, there is evidence that an excess DHA may be harmful: Greenland Inuits carry special isoforms of the Δ6 desaturase FADS2 that are particularly inefficient at desaturating PUFAs, hence limiting the amount of highly fluidizing LCPUFAs that they can produce, which may be adaptive to counter negative effects of a fish-rich diet [110].

3.3. Mechanisms that influence membrane composition

3.3.1. Kennedy pathway and Lands cycle

The specific composition of membrane phospholipids is the result of several complementary processes [96]. In particular, the composition of new PCs and PEs synthesized by the ER via the Kennedy pathway (using glycerol, acyl-CoA and either phosphocholine or phospho-ethanolamine as substrates) can be regulated by prior elongation or desaturation of FAs [111]. Selective transport of membrane components from the ER to other membranes via vesicles and lipid transport proteins can also affect the composition of different subcellular membranes [112,113]. Remodelling of phospholipids by FA chain exchange via the Lands’ cycle, which requires the action of diverse phospholipases [114,115] and ER-localized lysophospholipid acyl transferases [116,117], can also greatly influence the FA composition of intracellular membranes [103].

3.3.2. Composition of the fatty acid pool: metabolism and dietary uptake

De novo lipogenesis that produces the FAs used by the Kennedy pathway and Lands cycle has acetyl-CoA as a starting substrate which is elongated by a series of elongases and desaturated by desaturases. Three types of ER-localized desaturases exist in human: two Δ9 desaturases (SCD and SCD5), one Δ5 desaturase (FADS1) and one Δ6 desaturase (FADS2), which can introduce double bonds between carbons 9–10, 5–6 and 6–7, respectively, with the number 1 carbon being that of the carboxyl group. De novo lipogenesis is tightly coordinated with FA desaturation in adipocytes, and probably most cell types, such that the output is a balanced pool of SFAs and UFAs [118]. This is also true in the nematode *C. elegans* where the activity of desaturases is a key regulator of de novo lipogenesis [119]. The composition of the intracellular FA pool can also be regulated at the level of degradation; for example, several acyl-CoA synthetases preferentially activate medium-chain PUFAS (MCPUFAs) and long chain PUFAS (LCPUFAs) for import and degradation in mitochondria and peroxisomes, respectively [120,121], thus reducing the relative abundance of these PUFAs among the FAs available for incorporation into phospholipids.

Animals face a unique problem beyond management of their internal FA pools since most of their membrane phospholipids have a high turnover and are mostly assembled using dietary FAs. *C. elegans*, for example, replaces nearly 80% of its total membrane phospholipids

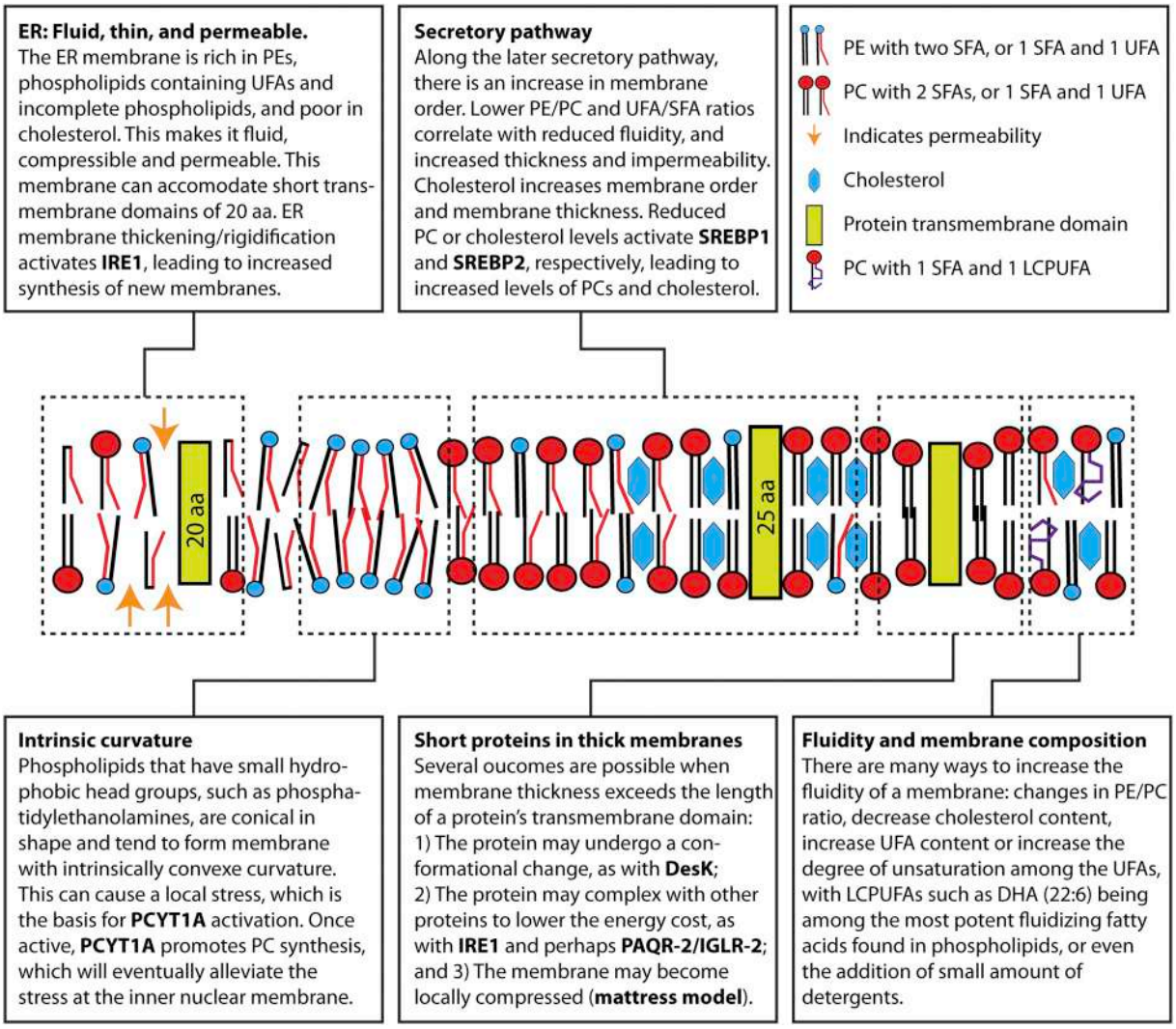


Fig. 5. Annotated model of a membrane with a variety of composition and properties illustrated as a continuum.

every day using mostly dietary fatty acids as building blocks [119]. On the other hand, mouse studies have shown that membrane phospholipid SFA content varies only very slightly even when the animals are fed diets varying vastly in their SFA content [122]. Effective mechanisms that compensate for dietary variation must therefore exist in animal cells in order to achieve the desired membrane properties. In mammals, for example, dietary SFAs may be desaturated via the Δ9 desaturase SCD to produce UFAs. SCD, in turn, contains a specific domain that insures its rapid turnover such that its abundance is mostly regulated by the transcription rate [123–125]. What then regulates the adaptive levels of SCD and other enzymes responsible for adjusting membrane composition? The answer lies, at least in part, in the existence of several sense-and-response proteins that detect membrane property defects and regulate compensatory responses. These will now be discussed in some details (an overview is provided in Fig. 7).

3.4. *MGA2 and the OLE1 pathway of Saccharomyces cerevisiae*

3.4.1. *Overview of the OLE1 pathway*

Like *B. subtilis*, *S. cerevisiae* possesses a single fatty acid desaturase, OLE1, which introduces a double bound in the Δ9 position of fatty acids esterified to CoA [53,126]. OLE1 localizes into the ER membrane, where most of the lipid biosynthetic machinery resides (Fig. 4). In this organelle, saturated C16:0 (palmitic acid) and C18:0 (stearic acid) acyl-CoA precursors are desaturated, yielding C16:1 (palmitoleic acid) and C18:1 (oleic acid), respectively, which are then distributed throughout the membranes of the cell systems and makes up more than 70% of the total fatty acids.

The *OLE1* gene is highly regulated in response to various environmental signals. Like the *B. subtilis des* gene, *OLE1* expression is transiently activated immediately after cooling [127]. It is also induced under hypoxic conditions [128]. Since the desaturation reaction utilizes oxygen as an electron acceptor, *OLE1* induction under hypoxia might be a response to UFA depletion under such limiting substrate conditions [129,130].

Insight into the molecular basis of how *OLE1* transcription is controlled has emerged from work by Zhang and Garfinkel [131]. Their studies identified two distantly homologous genes of the transcription factor NF-κB/Rel of higher eukaryotes, *SPT23* and *MGA2*, which are required for *OLE1* transcription. Disruption of either *SPT23* or *MGA2* has little effect on growth or UFA synthesis, whereas the simultaneous gene disruption results in synthetic auxotrophy for UFAs due to loss of *OLE1* mRNA [131]. From these data, the authors concluded that SPT23 or MGA2 activate OLE1 transcription. Although neither protein appeared to contain a recognizable DNA binding domain, they are thought to regulate transcription by modulating the local chromatin accessibility.

An understanding of the mechanism by which SPT23 and MGA2 activate *OLE1* expression came from studies in the S. Jenscht laboratory. SPT23 and MGA2 are initially synthesized as inactive precursors

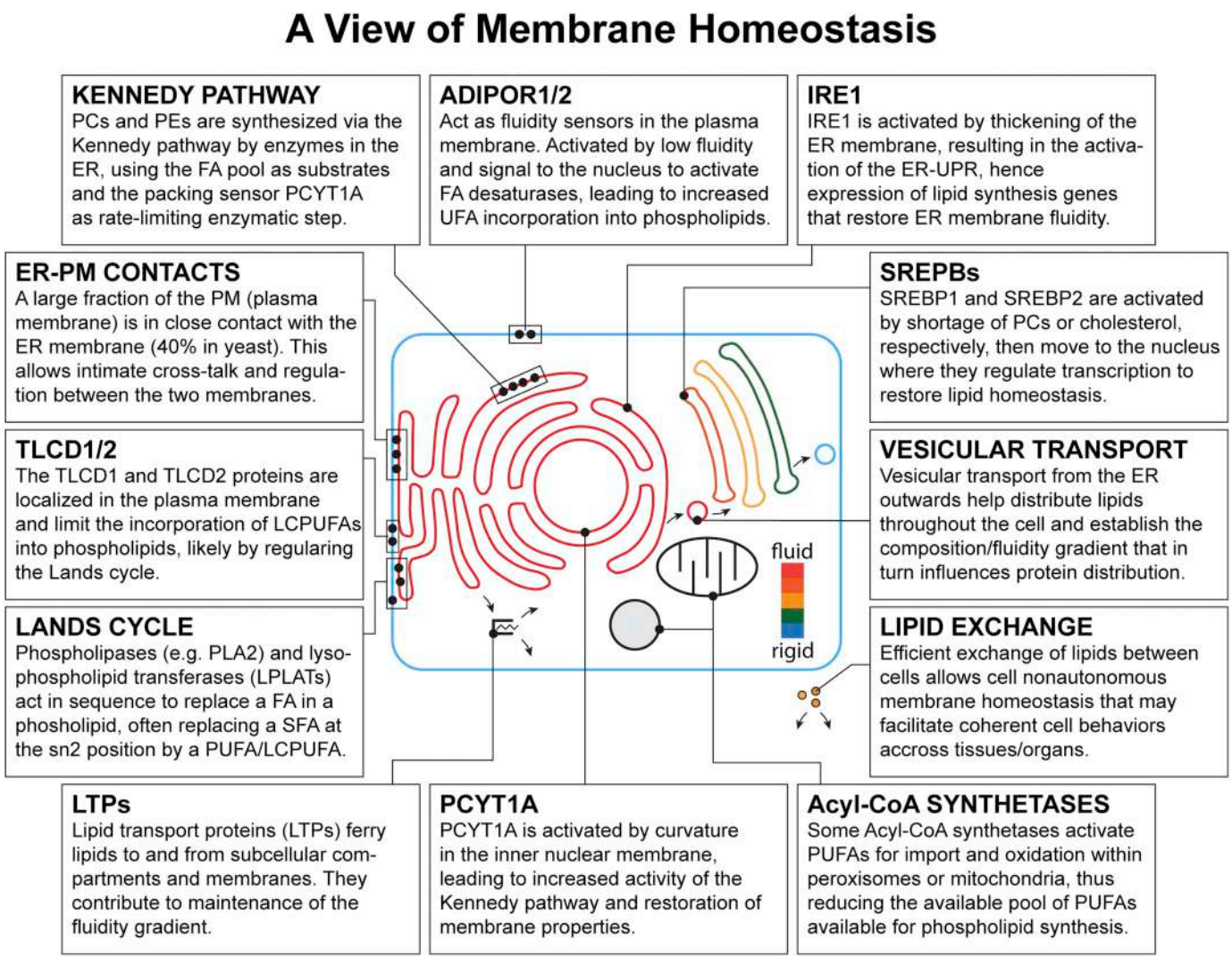


Fig. 6. A view of membrane lipid composition homeostasis. Cartoon representation of a cell with several features relevant to membrane homeostasis indicated.

that are anchored to the ER membrane via their single C-terminal transmembrane spans [132]. When UFAs are required for membrane biogenesis and function, MGA2 and SPT23 are released from the ER by proteolytic processing (Fig. 8). This process involves the ubiquitylation of the membrane-tethered precursor by the E3 ubiquitin ligase RSP5, ubiquitin chain remodeling, ATP-dependent transcription factor mobilization, and a processing step by the proteasome that clips the active transcription factor from its membrane anchor [132] (for a recent review see [133]). However, when UFAs are abundant, the ubiquitylation of MGA2 and SPT23 ceases, and the transcription factors remain tethered to the ER membrane. MGA2 and SPT23 regulate the expression of numerous abundantly expressed genes involved in ribosome biogenesis and lipid metabolism [134]. Interestingly MGA2, but not SPT23, activates the transcription of *ERG1*encoding for squalene monooxygenase, the first oxygen required step in ergosterol biosynthetic pathway [135]. Thus, MGA2 has also been implicated in mediating a hypoxic response. Despite this broad regulatory profile, synthetic lethality induced by the loss of MGA2 and SPT23 is reversed by supplementation of UFA to the growth medium [131], suggesting that OLE1 is the most critical target of these transcription factors.

Although MGA2 and SPT23 appear to be overlapping membrane sensors, a membrane-sensitive processing of SPT23 alone cannot fully account for *OLE1* regulation: cells lacking this transcription factor maintain a level of *OLE1* comparable to wild type cells [136]. In contrast, cells lacking MGA2 exhibit reduced levels of OLE1, resulting in a severely perturbed membrane lipid composition and ER stress. These and others observations identified MGA2 as the dominant transcription factor of the OLE pathway.

3.4.2. A possible sensing mechanism for MGA2

The observation that MGA2 exists as homodimeric, membrane-embedded precursor and that its proteolytic processing is controlled by exogenous UFA supplementation, suggested that the single TM helix (TMH) from MGA2 acts as a sensor of the lipid environment. To test the functional relevance of the TMH for membrane sensing and transcriptional factor activation, Covino et al performed a leucine scan along the predicted TMH of MGA2 [137]. To this end, each TMH residue was individually mutated to leucine and the respective construct was tested for transcription factor activation in vivo by determining the ratio of processed to unprocessed precursor. This approach led to the identification of two residues that are important for an efficient processing: a tryptophan and a proline located within the TMH. To gain insight into the dynamics of MGA2´s TMH and the role of these essential residues, coarse grained molecular dynamics simulations were performed with peptides corresponding to the TMH of MGA2 in two different membrane environments. The computational modelling simulation was complemented by structural dynamic of the N-terminal part of the TMH by site-directed spin labelling electron paramagnetic resonance spectroscopy [137]. Based on these evidences it was proposed that the sensor mechanism relies on highly dynamic homodimers of the TMH

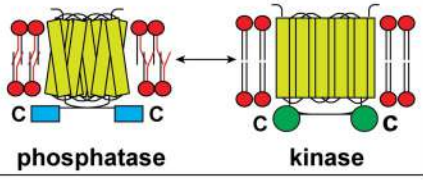
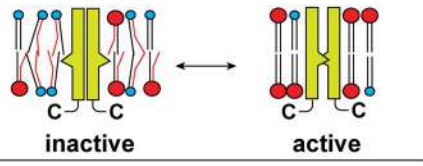
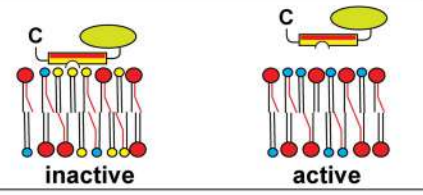
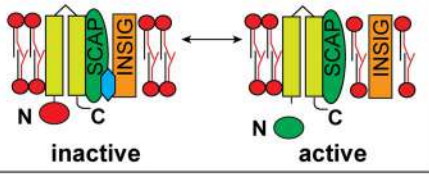
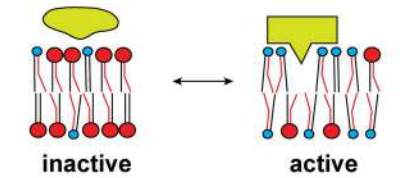
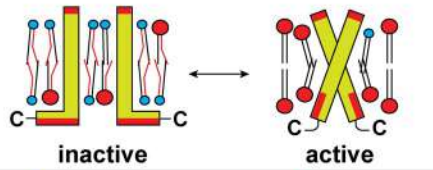
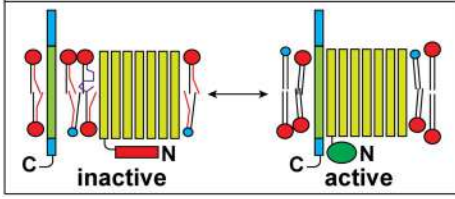
Membrane Property Sensor	Description
	**DesK dimer in plasma membrane (bacteria)** Membrane viscosity activates kinase conformation Effect: desaturase activation
	**Mga2 in ER (yeast)** Increased acyl chain packing promotes activating dimerization Effect: fatty acid desaturation via OLE1
	**Opi1 in ER (yeast)** Low phosphatidic acid in ER membrane releases Opi1 Effect: Opi1 suppresses synthesis of phospholipids, allowing restoration of phosphatidic acid levels.
	**SREBPs in ER (eukaryotes)** Cholesterol depletion leads to release of N-terminal bHLH domain SREBP1 effect: de novo lipogenesis/desaturation SREBP2 effect: mevalonate pathway of cholesterol synthesis
	**PCYT1A in inner nuclear membrane (eukaryotes)** Lipid packing defects allow activating interaction with membrane Effect: increased PC synthesis
	**IRE1 in ER (eukaryotes)** Membrane thickening leads to multimerization hence activation Effect: UPR activation, including de novo lipogenesis
	**PAQR-2 and IGLR-2 in plasma membrane (animals)** Membrane viscosity increases PAQR-2/IGLR2 interaction Effect: signal to promote desaturation

Fig. 7. General overview of membrane property sensors discussed in this review. See text for additional details.

and a tryptophan sensor residue conserved in MGA2 and SPT23. In this model, the TMHs rotate against each other and the population of distinct rotational states is determined by the interaction of the bulky tryptophan with the lipid environment. When the order of the lipid is decreased, the dimer is stabilized in a conformation where two sensing tryptophans point away from the dimer interface towards the lipids (Fig. 7). When the amount of UFAs decreases, promoting order among membrane lipids, the membrane stabilizes an alternative dimer configuration with the two tryptophans pointing towards the dimer interface in a pocket created by proline-induced kinks in the TMHs. This rotation hypothesis predicts that the structural dynamics of the TMHs is coupled to the ubiquitylation and activation of MGA2 (and likely SPT23). Clearly, much more experimentation is necessary to elucidate the mechanism of signal propagation from the site of sensing to the site of transcription factor ubiquitylation.

The specific lessons learned from the sensors DesK and MGA2 are a starting point to infer general principles of membrane fluidity sensors. They both use rotational motions in response to changes in the ordering of membrane lipids and both sensory TM segments need proline residues to link signal sensing to signal transduction. A key difference between the sensors is that while DesK responds to a bilayer hydrophobic mismatch [73,76] MGA2 is not sensitive to membrane thickness [137]. This is likely because the membrane of the ER is highly flexible to accommodate all type of newly synthesized proteins (Figs. 5 and 6) and the energetic cost of a hydrophobic mistmatch is low in an ER-like lipid environment [138]. Nevertheless, it is unlikely that the

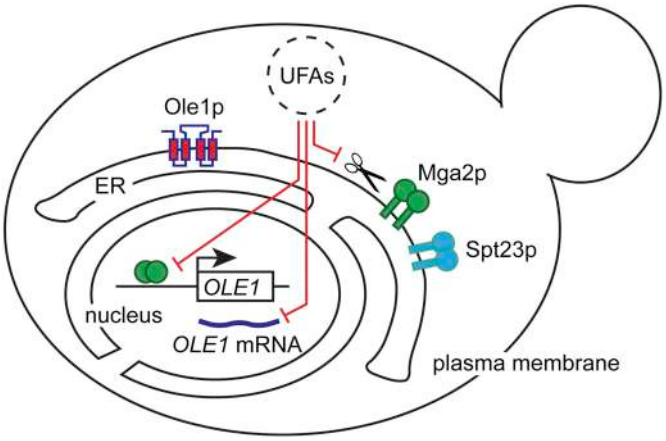


Fig. 8. Budding yeast desaturase gene expression requires proteolytic processing of either dormant transcription factors Mga2p or Spt23p. UFAs negatively downregulate OLE1 expression at multiple levels, including intramembrane proteolytic processing repression.

mechanisms of these sensors are unique and the way they respond to membrane properties can contribute to understand the sensing mechanism in a broad context of TM signal transductions.

3.5. OPI1: phosphatidic acid sensing

The yeast Opi1 is a soluble transcriptional repressor in *S. cerevisiae* that controls the expression of biosynthetic genes involved in the production of phospholipids derived from phosphatidic acid, namely PC, PE, phosphatidylinositol and phosphatidylserine [139,140]. In its inactive state, Opi1 is retained in the ER via interactions with specific lipid acyl chains phosphatidic acid lipids via its amphipathic helix [141–143], and also with the phosphatidic acid head group itself [144]. Elegant work from the Ernst group combined yeast genetics, molecular modelling and in vitro binding assays with liposomes of defined lipid composition to elucidate the mechanism of Opi1 regulation [144]. They showed that when phosphatidic acid levels are sufficient to meet phospholipid biosynthetic needs, Opi1 is retained in the ER membrane through its association with phosphatidic acid via a specialized amphipathic helix that lies flat against the ER membrane and tightly grips the phosphatidic acid head group, hence preventing its entry into the nucleus. Upon phosphatidic acid shortage, Opi1 is soluble and enters the nucleus where it represses target genes, hence allowing the phosphatidic acid levels to rise again [144]. Opi1 is therefore a prime example of a sense-and-response protein that specifically monitors and adjusts the abundance of phospholipids with a specific headgroup, namely the phosphate head group in phosphatidic acid.

3.6. SREBP1/SREBP2: cholesterol and PC sensing

The sterol regulatory element-binding proteins (SREBPs) are dual function lipid composition sensors/transcription factors of the bHLH leucine zipper family that act as “nodes of convergence” for many signaling networks to control lipid metabolism and homeostasis [145,146]. Two SREBPs exist in mammals: SREBP2 that senses cholesterol levels and regulates cholesterol synthesis genes, and SREBP1 (isoforms 1a and 1c) that primarily senses phospholipid composition and regulate both lipogenesis and cholesterol synthesis genes [146]. Silencing of both genes in breast cancer cells leads to dramatic reduction in the expression of many genes important for fatty acid de novo synthesis (e.g. SLC25A1 and ACLY) or desaturation (SCD, FADS1 and FADS2), accompanied by excess of SFAs in phospholipids and activation of the unfolded protein response (UPR), an ER response to membrane stress of misfolded proteins (see the section on IRE1) [147].

The SREBP2 pathway is one of the first and best understood involving sense-and-response proteins that are activated by specific membrane composition defects, namely cholesterol shortage, and inactivated when the defect is corrected [145,148]. SREBP2 is synthesized and initially resides in the ER as a two-transmembrane domain protein, with its C and N termini extending into the cytoplasm, and is associated with the regulatory proteins SREBP cleavage-activating protein (SCAP) and insulin-induced gene protein, which comes in two isoforms, INSIG1 and INSIG2. INSIG1 expression is dependent on SREBP and it is also rapidly degraded when cholesterol levels are low while INSIG2 is always expressed but at low levels. SCAP is an 8-transmembrane domain protein that can, when ER cholesterol levels are low, promote transport of the SREBP-SCAP complex towards the Golgi via COPII-binding, hence recruitment into COPII vesicles. Once in the Golgi, the SREBP-SCAP complex is retained by the seven transmembrane domain PAQR-3 [149], and SREBP is proteolytically processed by site-1 protease (S1P) and site-2 protease (S2P), which releases the bHLH leucine zipper transcription factor domain that can enter the nucleus and interact with the mediator subunit MED15 to regulate target genes containing sterol regulatory elements (SREs) in their regulatory regions, including the HMGCR gene that encodes the rate limiting enzyme for cholesterol synthesis [150] (Fig. 7). Transport of the SREBP-SCAP complex from the ER to the Golgi is inhibited by sufficient cholesterol levels: a large ER luminal loop within SCAP preferentially interacts with cholesterol, which then prevents interaction between SCAP and COPII, hence leading to retention of the SREBP/SCAP complex within the ER via their association with the ER-retention protein INSIG. Cholesterol production is regulated in yet an additional way by INSIG, which interacts in a cholesterol-dependent manner with the ER membrane resident HMGCR enzyme to promote its ubiquitination and degradation only when cholesterol levels are sufficient [151]. Thus, INSIG regulates HMGCR at two levels, both of which are cholesterol-dependent: 1) control maturation of active SREBP required for HMGCR transcription and 2) control HMGCR protein degradation.

The regulation of SREBP1 is less well understood. SREBP1 promotes lipogenesis and fatty acid desaturation in response to PC depletion, a regulation that is conserved from nematodes to mammals [152,153] and involves Arf1-dependent processes. SREBP1 proteolytic activation is also inhibited by PUFAs that may cause a redistribution of cholesterol [154–156] or prevent INSIG1 degradation hence promote retention of the SREBP1-SCAP complex in the ER [157]. SREBP1 mRNA levels are also lowered by PUFAs, suggesting regulation also at the level of transcription [158]. Altogether, the accumulating evidence suggests that SREBP1 is activated by lipid composition defects (PC or PUFA shortages) that are corrected by activation of its downstream target genes.

Additional levels of lipid-dependent SREBP regulation exist besides their proteolytic activation. In particular, several nuclear hormone receptors, such as hepatocyte nuclear factor 4 (HNF4) or the liver X receptor (LXR) that that activated by lipid ligands, regulate SREBP activity via direct binding and thus provide another level of regulation to maintain lipid, hence membrane, homeostasis [159,160].

3.7. PCYT1A and tafazzin: sensing membrane packing defects

PC is the most abundant phospholipid of eukaryotic cell membranes comprising 30%–60% of total phospholipids and it stands to reason that its synthesis be regulated to meet demand, for example during cellular growth. The rate-limiting step in the Kennedy pathway of PC synthesis is catalysed by cytidine triphosphate:phosphocholine cytidyltransferase (PCYT1A, aka CCT). Structurally, PCYT1A orthologs from yeast to humans contain a catalytic α/β fold followed by a membrane binding region and a disordered C-terminal tail [161]. PCYT1A also contains a regulatory amphipathic helix that associates with PC-containing bilayers with a high content of conical lipids (e.g. PEs): membrane association rapidly facilitates PCYT1A catalytic activity by promoting an

unstructured loop to fold into a helix causing removal of an adjoining helix that otherwise prevents substrate access to the catalytic pocket of the dimeric enzyme [161]. Once the levels of PCs are high enough to restore sufficient membrane packing, the PCYT1A amphipathic helix is repelled from the membrane, returning PCYT1A to its inactive state [162,163] (Fig. 7). Recently, it was shown that PCYT1A translocates onto the inner nuclear membrane, which is continuous with the ER, in response to increased membrane packing defects in the nuclear lumen-facing leaflet, which reflects a shortage of PCs and results in curvature stress in yeast, fly, and mammalian cells, and that this activates PCYT1A until PC levels are restored [164]. The inner nuclear membrane is therefore the site of membrane-composition sensing and enzymatic activity for PCYT1A.

Like PCYT1A, the mitochondrial membrane resident tafazzin is also an amphiotropic enzyme, i.e. regulated by a membrane ligand: it is an acyl transferase that incorporates PUFAs into cardiolipin (composed of two phosphatidic acids connected to a glycerol backbone) or lysophospholipids only when activated by packing defects in the curved membranes of mitochondria or in vitro assembled vesicles of defined compositions [165]. Studies using defined lipid vesicles also showed that the tazaffin substrate specificity, i.e. which acyl chains will be incorporated into which phospholipids, is also determined by the specific packing defects of the vesicles [165]. There exist many proteins that are regulated by membrane packing defects, including cytosolic proteins containing amphipathic membrane anchors that depend on packing defects for their recruitment to ER, autophagosomes or Golgi [104,166–170]. What is special about PCYT1A and tafazzin is that their activation by the packing defects will lead to the removal of these same defects.

3.8. IRE1: sensing unfolded protein and ER membrane stress

The unfolded protein response (UPR) is an ER stress response pathway initially discovered as a response to the accumulation of unfolded proteins in the ER. Such accumulation triggers the multimerization of the ER membrane resident protein IRE1, hence activating it. IRE1 is a type I transmembrane protein composed of an N-terminal ER luminal domain, a transmembrane segment and a cytoplasmic domain containing kinase and RNAse activities. The crystal structures of several IRE1 domains have been resolved and explain the mechanism of its activation through oligomerization. The ER-luminal domain has a triangular structure with beta-sheets forming each of the sides, one of which mediates dimerization of the luminal domain when unfolded proteins are present [171]. Dimerization leads to trans-autophosphorylation that promotes further oligomerization of IRE1, leading to self-assembly of the cytosolic region into a helical rod structure with RNAse activity, i.e. the active form of IRE1 [172,173]. Activated IRE1 in turn promotes an alternative splicing of the XBP1 transcript, a basic leucine zipper transcription factor of the ATF/CREB family that stimulates transcription of hundreds of ER response genes, including lipid biosynthesis genes that enlarge the ER hence increasing its capacity [174,175]. Several observations have shown that the UPR can be regulated directly by membrane stress and independently of misfolded proteins. For example, membrane expansion can alleviate ER stress without the activation of the chaperones that are typically part of the ER-UPR [176], and defects in lipid metabolism genes or in fatty acid shape can modulate the ER-UPR even in the absence of misfolded proteins [177,178]. The key sensor of ER membrane defects is IRE1 [179,180], which therefore has dual sensing functions but one downstream response pathway, namely activation of the UPR via XBP-1.

Various membrane rigidifying perturbations can cause IRE1 activation: high PE to PC ratio [181], increased lipid saturation [136], increased sterol levels [182], and inositol depletion required for the synthesis of phosphatidylinositol (PI) and sphingolipids [179]. In *C. elegans*, IRE-1-dependent UPR activation is also triggered by inhibition of the mevalonate pathway, which prevents prenylation of small GTPases important for vesicular trafficking [183,184], and by perturbation of lipid metabolism that cause excess SFAs or altered PE/PC ratio [185]. Altogether, these observations suggest that IRE1 senses a general ER membrane property, such as thickness, rather than a narrow specific property or the levels of a specific lipid type.

IRE1 contains an ER lumen-facing amphipathic helix continuous with one short transmembrane hydrophobic helix attached to a hydrophilic region on the cytosolic side. The amphipathic helix tends to lie parallel to the membrane, whereas the transmembrane helix prefers a perpendicular orientation, forcing the transmembrane domain to switch between a straight, tilted conformation and a kinked “L-shaped” conformation as suggested by extensive molecular dynamics simulations. The transmembrane hydrophobic helix is particularly short and constrained by the polar residues on the cytosolic side and by the amphipathic helix on the other. This causes a local squeezing of the bilayer, that disrupts the lipid acyl chain order. These perturbations come with an energetic penalty that can drive the clustering of the transmembrane helix upon lipid bilayer stress, promoting both dimerization and oligomerization of dimers. Lipid compositions that lead to a more ordered and thicker membrane, such as being rich in SFAs, tend to resist compression and will therefore promote the formation of these clusters, which lowers the average energy cost of membrane compression per IRE1 protein (Fig. 7). IRE1 clustering promotes autophosphorylation, further oligomerization and formation of the active RNAse rod-like domain capable of splicing, hence activating, the XBP1 mRNA. Under unstressed conditions, the ER membrane is very fluid and the energetic penalty for bilayer squeezing is lower and no clusters of IRE1 will form unless they are stabilized by accumulating unfolded proteins [133,186]. The example of IRE1 highlights how a single sensor protein can respond to various perturbations of the sterol, sphingolipid, fatty acid, and glycerophospholipid metabolism by sensing collective bilayer properties, namely thickness/compressibility. On an evolutionary scale, it seems that the accretion of distinct sensing functions (activation by unfolded proteins or by ER membrane stress) into one protein, IRE1, was advantageous since the same downstream response, UPR, addresses both types of stress by producing fresh ER membranes, which increase the processing capacity of the ER.

3.9. PAQR-2 and adipors: sensing membrane fluidity in animal cells

3.9.1. PAQR-2 maintains membrane fluidity in C. elegans

The plasma membrane-localized putative adiponectin receptors AdipoR1 and AdipoR2 are members of the evolutionarily conserved PAQR (named so after the founding members, Progestin and AdipoQ Receptors) family of proteins characterized by seven transmembrane domains with their N-terminus facing the cytoplasm [187]. The crystal structure of the AdipoR transmembrane domains has been resolved and revealed a barrel shaped helical bundle with a rather large internal cavity partially opened on the cytoplasmic side, as well as a zinc-coordinated center that may be a catalytic site located within the barrel, near the cytoplasmic surface (Fig. 9; one of the shown structures contains a C18:0 fatty acid trapped within the helical bundle). Most PAQR proteins likely act as hydrolases with different specificities [188], and the structure of the AdipoRs suggests that they too are likely hydrolases, and perhaps specifically ceramidases [189–191], which was confirmed by enzymatic assays [191] and is conserved in yeast homologs [192,193]. The ceramidase activity, which would produce a signaling sphingosine 1-phosphate as well as a putative fatty acid that may also serve as a signal, has been proposed to mediate the well-established anti-diabetic effects of the AdipoRs [194,195]. However, in what contexts is AdipoR activity required and what is the cellular consequence of this activity? Recent findings in *C. elegans* have begun answering these important questions.

The nematode *C. elegans* contains five proteins that belong to the PAQR family of proteins, including PAQR-1 and PAQR-2 that are highly homologous to the mammalian AdipoR1 and AdipoR2 proteins [196].

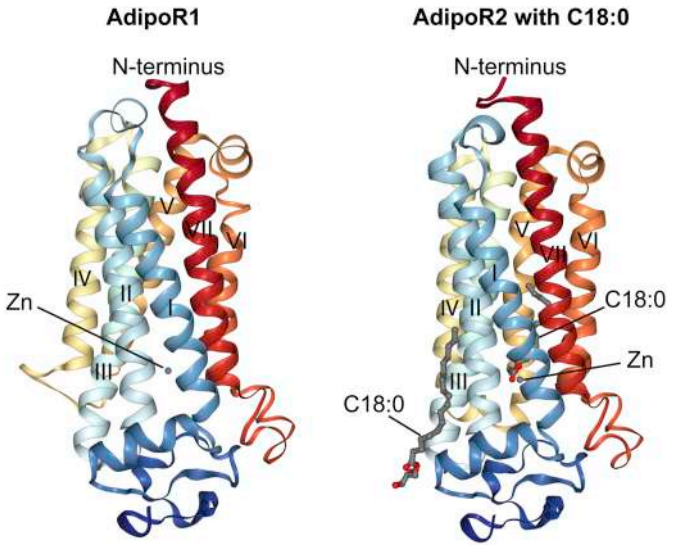


Fig. 9. Structures of AdipoR1 and AdipoR2. AdipoR1 structure with the zinc ion within the barrel-shaped cavity indicated. AdipoR2 is shown in complex with a C18 free fatty acid within the internal cavity and zinc ion indicated (another C18:0 is shown to the left of the structure). Note that AdipoR1 and AdipoR2 are shown with different orientations. Images of the transmembrane domains of AdipoR1 (PDB ID: 5LXG; glutamine 100 to serine 381) and AdipoR2 (PDB ID: 5LXA; glutamine 89 to glycine 368) were obtained from PDB IB [222] based on published crystal structures [190,191]; the structures of the N-terminal cytoplasmic domains has not been solved and is not shown.

Several lines of experimental evidence indicate that PAQR-2 is a regulator of membrane fluidity essential for adaptation to membrane rigidifying conditions. In particular, mutant worms lacking a functional PAQR-2 protein have a morphological defect in the fragile membranous structure that is the tail tip [196,197] and are intolerant of conditions that promote membrane rigidification, such as cold or diets that increase saturated fatty acid (SFA) content in the worms [196,198–200]. For example, pre-loading the dietary *E. coli* with the SFA palmitate or including small amounts (5 mM) of glucose in the culture plate (which is then converted to SFAs by the dietary *E. coli*) results in increased SFA content in membrane phospholipids of the *paqr-2* mutant as measured using mass spectroscopy analysis (i.e. lipidomics), membrane rigidification as measured using Fluorescence Recovery After Photobleaching (FRAP) [25], and death of the *paqr-2* mutant (the growth and membrane fluidity of wild-type worms is unaffected by palmitate or glucose) [198,199]. The *paqr-2* mutant defects can be suppressed genetically by mutations in other genes that result in increased production of unsaturated fatty acids (UFAs) accompanied by normalization of plasma membrane fluidity or, alternatively, by cultivation in the presence of fluidizing amounts of non-ionic detergents such as Triton X-100 or NP-40 [199,200].

Forward genetic screens in *C. elegans* have led to the identification of two broad classes of mutations that can suppress the membrane fluidity defects in *paqr-2* mutants [200–202]. One class includes several mutations that promote fatty acid desaturation while the other class includes alleles of the novel gene *fld-1* (discussed below) that promote incorporation of LCPUFAs into phospholipids. The best suppression of *paqr-2* mutant phenotypes is achieved by combining mutations from both classes, suggesting that the complete *paqr-2* membrane homeostasis program includes promotion of FA desaturation and channeling of the resulting PUFAs into phospholipids [202]. The normal function of PAQR-2 is therefore to regulate fluidity by adjusting the UFA/SFA ratio within phospholipids (see model in Fig. 10), which is a fundamental mechanism of cold adaptation in many poikilotherms [203–205] and provides protection against the rigidifying effects of dietary SFAs [198].

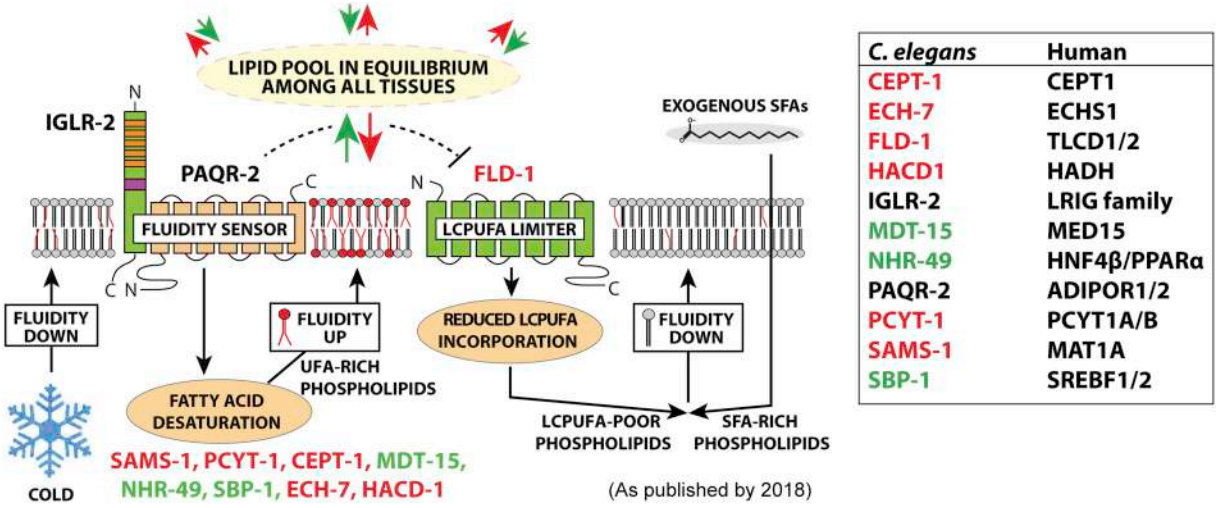
3.9.2. The AdipoRs maintain membrane fluidity in mammalian cells

The high degree of sequence homology between PAQR-2 and the AdipoRs (53.7% amino acid identity with AdipoR2 over a 283 aa region) suggests that these proteins have the same cellular functions, i.e. act as sensors/regulators of membrane properties. This has now been demonstrated for the mammalian AdipoRs: the AdipoR1 and AdipoR2 proteins also act as regulators of membrane fluidity that counter the rigidifying effects of exogenous SFAs in at least four human cell types by maintaining levels of UFAs among PE and PC phospholipids in a SCD-dependent manner [198,202,206,207]. This shows that the mammalian AdipoRs also respond to membrane rigidification by promoting FA desaturation. Regulation of membrane fluidity is therefore an evolutionary conserved function of the PAQR-2/AdipoR proteins that is essential to cellular health in the presence of exogenous SFAs.

3.9.3. Fluidity sensing and signaling mechanisms by PAQR-2/AdipoRs

The molecular mechanism by which PAQR-2 senses and responds to changes in membrane fluidity are mostly unknown. The mammalian homologs, the AdipoRs, are thought to functionally interact with APPL1, a cytosolic protein that has been called the “missing link” in the adiponectin-signaling cascade that may transmit signals from the AdipoRs to proposed downstream targets such as AMPK or p38MAPK [208,209]; however, no APPL1 homolog has been identified in *C. elegans*, and few independent reports have confirmed the role of APPL1 in AdipoR signaling. In an effort to discover PAQR-2 interactors, an unbiased forward genetics screen was performed to identify *paqr-2* genocopiers, i.e. other genes that, when mutated, produce the same phenotypes as mutations in the *paqr-2* gene. This screen led to the identification of two new alleles of *paqr-2* itself and, most importantly, three alleles of a gene called *iglr-2*, which is the only true *paqr-2* genocopier identified to date [199]. IGLR-2 is predicted to consist of an intracellular C-terminal domain, a single transmembrane domain, and an extracellular region containing an immunoglobulin (Ig)-like domain and several leucine rich repeats (LRRs) [210]. The sequence and domain structure of IGLR-2 is related to that of nearly forty mammalian LRIG-type membrane proteins with a range of expression patterns and functions [211]. IGLR-2 is a protein essential for PAQR-2 function: the two proteins are co-expressed strongly in the plasma membrane (most clearly in the gonad sheath cells when seen using GFP reporters), physically interact with each other as determined using bifluorescence complementation (BiFC) and give identical phenotypes when mutated [199].

One intriguing possibility regarding the fluidity sensing mechanism is that membrane thickness or fluidity properties can influence the conformational state of the PAQR-2/IGLR-2 complex. Quite speculatively, rigid membranes could cause IGLR-2 to interact in such a way as to displace the regulatory intracellular domain of PAQR-2, thus opening the cytoplasm-facing channels for in/out flow of hydrolytic substrates and products. The cytoplasmic N-terminal domain does not appear to have a very specific intrinsic function for at least three reasons: 1) it is the least conserved region of the protein [196]; 2) it is predicted to be a mostly disordered region [212]; and 3) inserting a GFP coding sequence in the middle of the N-terminal cytoplasmic domain of PAQR-2 does not affect its function [196]. That the N-terminal domain acts primarily as a lid regulating access to the barrel-like channel formed by the transmembrane domains would be in agreement with the crystal structure of the AdipoRs which indicates that a large opening on the cytoplasmic side of the AdipoRs can be formed between helices III-VII if the N-terminal region is displaced [190]. In this context it is interesting that human PAQR3, a protein with sequence homology to the AdipoRs and the *C. elegans* PAQR-2, acts as a Golgi retention signal for the SREBP-SCAP complex with which it interacts via its cytosolic N-terminal domain in conditions of low cholesterol, hence promoting protease-dependent activation of SREBP [149]. The interaction between PAQR3 and SREBP-SCAP is inhibited by high cholesterol levels, which instead favor interaction of SREBP-SCAP with INSIG, hence



C. elegans	Human
CEPT-1	CEPT1
ECH-7	ECHS1
FLD-1	TLCD1/2
HACD1	HADH
IGLR-2	LRIG family
MDT-15	MED15
NHR-49	HNF4β/PPARα
PAQR-2	ADIPOR1/2
PCYT-1	PCYT1A/B
SAMS-1	MAT1A
SBP-1	SREBF1/2

Fig. 10. Model of membrane fluidity regulation by PAQR-2 and IGLR-2. Proteins that can act as PAQR-2 and IGLR-2 suppressors are colored in red (loss-of-function mutations) or green (gain-of-function mutations), and the name of the human homologs are listed in the table to the right. PAQR-2 and IGLR-2 act together as a membrane fluidity sensor, with loss of fluidity due to cold temperature or excess SFA within phospholipids acting as an activating condition for the complex. Once activated the PAQR-2/IGLR-2 complex signals, perhaps via a hydrolytic (e.g. ceramidase or other) activity that releases a lipid signalling molecule, to promote fatty acid desaturation by upregulating expression of the Δ9 desaturases FAT-6 and FAT-7. A likely target of PAQR-2 signalling is the nuclear hormone receptor NHR-49, though PAQR-2 may also act via the other targets identified as suppressors. FLD-1 mutations can also promote membrane fluidity and act as PAQR-2/IGLR-2 suppressors since a natural function of the protein is to limit the insertion of LCPUFAs into phospholipids; mutating FLD-1 therefore leads to an increase in LCPUFA-containing phospholipids, which improves fluidity. Membrane homeostasis is cell nonautonomous, indicating efficient exchange of lipid pools among *C. elegans* tissues and organs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

retention in the ER where SREBP is inactive. Thus, the regulatory PAQR-3 interaction with SREBP-SCAP is dependent on membrane composition and occurs through the N-terminal cytosolic domain, just as the proposed regulatory interaction between IGLR-2 and the N-terminal domain of PAQR-2 may be dependent on the fluidity of the plasma membrane. Other regulatory PAQR-2 interactions could also be influenced by membrane properties. In particular, it is possible that clusters of PAQR-2 proteins or of PAQR-2/IGLR-2 complexes form under conditions of membrane rigidity, which would be analogous to the IRE1 multimerization under conditions of ER bilayer stress [186] and consistent with the detection of AdipoR1 dimers in mammalian cells [213].

In any case, it would seem that the PAQR-2/IGLR-2 complex senses a general rather than specific membrane property. That is because the *paqr-2* and *iglr-2* mutants are sensitive to various types of rigidifying challenges (e.g. cold or SFA-rich diets) and their phenotypes can be suppressed by a variety of ways (e.g. exogenous UFA supplements, mutations that promote desaturation, detergents or small increases in the abundance of LCPUFAs among phospholipids). Altogether these observations suggest that the PAQR-2/IGLR-2 senses a rather general membrane property, conveniently termed fluidity, rather than a discrete property or lipid type.

3.9.4. Are PAQR-2 and the AdipoRs adiponectin receptors?

No *C. elegans* homolog of adiponectin has so far been identified either via homology searches or genetic screens for mutants that mimic or suppress the *paqr-2* mutant phenotypes [196,197,199,202]. Furthermore, the PAQR-2 protein in *C. elegans* is still functional when a GFP fusion partner is fused to the short extracellular C-terminus of the protein [196], which could disrupt binding to a ligand [190]. It therefore appears that PAQR-2 does not require an adiponectin-like ligand for its function as a membrane composition regulator in *C. elegans*. Similarly, all experiments demonstrating the function of the AdipoRs as regulators of membrane fluidity in human cells were carried out in the absence of adiponectin [207]. We therefore conclude that the AdipoRs can function as fluidity regulators independently of adiponectin. However, it does remain a possibility that adiponectin, or an adiponectin-like protein in *C. elegans*, can in a non-essential way modulate the activity of the AdipoRs, or of PAQR-2 in *C. elegans*.

3.10. TLCD1/2 AND FLD-1: limit PUFA-containing phospholipids in animal cells

Eight mutant alleles of the gene *fld-1* (membrane *fl*uid*d*ity homeostasis-1) were isolated in a screen for suppressors of dietary SFA intolerance in *paqr-2* mutants [202]. *fld-1* encodes a multiple-transmembrane domain protein homologous to the human TLCD1 and TLCD2 proteins that belong to a protein family characterized by a TLC (TRAM, LAG1 and CLN8) domain. The *C. elegans* FLD-1 and the mammalian TLCD1/2 are localized to the plasma membrane where their normal function appears to limit the generation of potent membrane fluidizing LCPUFA-containing phospholipids [202]. Mutations or inhibition in FLD-1/TLCDs protects against SFA rigidification because it allows higher levels of LCPUFA-phospholipids to accumulate in membranes, hence improving fluidity (see model in Fig. 10). A possible mechanism of action for the TLCD1/2 and FLD-1 proteins is that they regulate the Lands cycle to promote replacement of FAs at the sn-2 position of phospholipids by LCPUFAs. Extensive contacts exist between the ER and the plasma membrane [214–216] and could allow the TLCD1/2 and FLD-1 proteins localized in the plasma membrane to influence the activity/specificity of ER-resident acyl transferases.

3.11. Cell nonautonomous membrane homeostasis in animals

Fascinatingly, the regulation of membrane homeostasis by *paqr-2* and AdipoR2 is cell non-autonomous (see model in Fig. 10). Several lines of evidence support the cell nonautonomous nature of membrane homeostasis [206]: 1) Genetic mosaics in *C. elegans* that express PAQR-2 or IGLR-2 only in intestine or hypodermis become systemically rescued; 2) Tissue-specific promoters can drive PAQR-2 expression specifically in intestine, muscle or hypodermis and still can rescue membrane fluidity systemically (i.e. throughout the whole animal) and in a manner that requires the expression of desaturases in the PAQR-2 expressing tissue; and 3) Human HEK293 cells that express AdipoR2 can

help maintain membrane fluidity in AdipoR2-deficient, SFA-challenged cells in the absence of any physical contact [206]. The transport of lipids among *C. elegans* tissues is likely mediated via the pseudocoelomic fluid and may involve vitellogenins, other lipoprotein-like proteins such as DSC-4, or other pathways [217]. That lipids are in constant flux among *C. elegans* tissues should come as no surprise given that nearly 80% of phospholipids are replaced daily in an adult *C. elegans*, mostly using dietary fatty acids as building blocks and thus implying extensive trafficking between the intestine and other tissues. Similarly, while the most direct way for phospholipids exchange between mammalian cells or cellular compartments is through membrane contact sites [218], several contact-independent mechanisms also exist. Albumin is the major long-range transport system for unesterified FAs, which make up < 10% of blood fatty acids [219]. Fatty acid esters, which make up > 90% of blood fatty acids (i.e. triacylglycerol, cholesterol esters and phospholipids), are transported in blood by lipoprotein particles homologous to the *C. elegans* vitellogenins. These particles are made of a core consisting of a droplet of triacylglycerols and/or cholesteryl esters and a surface monolayer of phospholipid, cholesterol and specific proteins (apolipoproteins), e.g. Apolipoprotein B-100 in the case of LDL. Phopholipid transfer proteins are also ubiquitously expressed and can facilitate the transfer of phospholipids and other lipids between cells and tissues [220]. Physiologically, there are clear benefits to cell non-autonomous maintenance of membrane homeostasis, including achieving “ordered heterogeneity”, a fundamental property of biological systems whereby tissues achieve healthy homogeneity even though they are composed of heterogeneous cells experiencing various levels of stresses or mutations [221]. It will be interesting to explore the relevance of these findings for human physiology. For example, tumor cells with abnormal lipid metabolism could affect the membrane composition of neighboring healthy cells. Conversely, the cell non-autonomous nature of membrane homeostasis may contribute to the robustness of whole organisms in response to dietary or disease state challenges: impaired tissues or cells may continue to have functional membranes because of healthy neighbors.

4. Concluding remarks

Membrane homeostasis is robust because many sense-and-response mechanisms monitor membrane properties and adjust its composition. Furthermore, many of the membrane sense-and-response proteins seem to have accreted multiple strategies that contribute to their effectiveness. For example, IRE1 senses both misfolded proteins and membrane defects in the ER, while INSIG regulates HMGCR levels both transcriptionally via SREBP and post-translationally by regulating its degradation. It is also interesting that many of the membrane defect sense-and-response proteins identified so far are activated by membrane rigidification. This is certainly true for DesK, Mga2, IRE1 and the PAQR-2/IGLR-2 complex. This suggests that the most common challenge to membrane homeostasis is excess rigidity. On the other hand, several sensors detect specific defects or lipids: PCYT1A and the acyl transferase tafazzin (activated by packing defects) and the Opi1 and SREBPs that are activated by depletion of a specific lipid (phosphatidic acid and cholesterol, respectively) or by PC shortage (in the case of SREBP1). It will be interesting in the future to see if sensors exist that react to excess fluidity or that monitor the levels of other phospholipid types (e.g. cardiolipins in bacteria, and ceramides, phosphatidylserines, etc in eukaryotes) or properties (e.g. charge, bilayer asymmetry, etc). Finally, a deeper analysis of protein structures in different membrane contexts is necessary before we have a satisfying understanding of how the many sense-and-response proteins maintain membrane homeostasis across all forms of life and environments.

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Declaration of competing interest

The authors declare that they have no conflicts of interest.

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