Membrane Fluidity Regulation:
From *C. elegans* to mammalian cells

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SVANENMÄRKET

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To my beloved Mother Sai,

ABSTRACT

Biological membranes are primarily an assembly of lipids and proteins. Collectively, these constituents within a phospholipid bilayer determine the physical membrane properties such as fluidity, viscosity, thickness, packing and permeability. Maintenance of membrane properties within acceptable margins, i.e. membrane homeostasis, is fundamentally important for cellular processes. For example, it is a well-established phenomenon that poikilothermic organisms, that cannot control their body temperature, constantly adapt their membrane lipid composition in order to maintain optimal membrane fluidity for membrane functions in spite of variation in ambient temperatures. Regulatory mechanisms must also exist in mammals to maintain membrane lipid heterogeneity across the secretory pathway and to compensate for dietary lipid variation. However, the molecular mechanisms of such an adaptive response in mammals remain poorly understood. Here, using systematic genetics, lipidomics and membrane property assays, we have established that the PAQR-2/IGLR-2 complex in *C. elegans* and AdipoR2 (a PAQR-2 homolog) in mammalian cells specifically respond to the toxic membrane-rigidifying effects of dietary saturated fatty acids (SFAs) and promote fatty acid desaturation to restore membrane composition and fluidity.

In an attempt to understand other mechanisms essential to prevent SFA-mediated cellular toxicity, we also performed an unbiased forward genetic screen in *C. elegans*. Strikingly, this screen for SFA-tolerance genes led only to the isolation of novel *paqr-2* and *iglr-2* alleles; this strongly indicates that *paqr-2* and *iglr-2* are important genes specifically essential to respond to toxic effects of dietary saturated fats. In particular, we noted that in worms and cells that lack PAQR-2/AdipoR2 function, exogenous SFAs becomes rapidly incorporated into membrane phospholipids, leading to membrane rigidification. This was accompanied by an abnormal transcriptional response, impaired mitochondrial respiration and increased ER-UPR as measured in HEK293 cells. Interestingly, we noticed that the toxic effects of exogenous SFAs can be completely mitigated by supplying the cultured cells with small amounts of membrane fluidizing unsaturated fatty acids (UFAs). Consistently, we also found that facilitating the accumulation of UFAs either with mutations in *fld-1* in worms or silencing the *fld-1* mammalian homologs TLCD1/2, which normally function to limit the incorporation of polyunsaturated fatty acids in membrane phospholipids, is protective and able to attenuate SFA-mediated cellular toxicity. Altogether, these results suggest that maintenance of an optimal SFA/UFA ratio is crucial for normal cellular function and that the PAQR-2/AdipoR2 proteins essentially act as "**guardians of membrane homeostasis**".

Keywords: PAQR-2, adiponectin receptors, IGLR-2, saturated fats, membrane fluidity, fatty acid desaturation, unsaturated fatty acid, membrane homeostasis

PUBLICATIONS

This thesis is based on the following publications (* indicate shared first authorship).

I. ***Caenorhabditis elegans* PAQR-2 and IGLR-2 Protect Against Glucose Toxicity by Modulating Membrane Lipid Composition**
Svensk E., <u>Devkota R.</u>, Ståhlman M., Ranji P., Rauthan M., Magnusson F., Hammarsten S., Johansson M., Borén J., Pilon M.
PLoS Genetics 2016

II. **The Adiponectin Receptor Adipor2 and its *Caenorhabditis Elegans* Homolog PAQR-2 Prevent Membrane Rigidification by Exogenous Saturated Fatty Acids**
<u>Devkota R.</u>*, Svensk E.*, Ruiz M., Ståhlman M., Borén J., Pilon M.
PLoS Genetics 2017

III. **Membrane Fluidity Is Regulated Cell Non-autonomously by *Caenorhabditis elegans* PAQR-2 and its Mammalian Homolog AdipoR2**
Bodhicharla R.*, <u>Devkota R.</u>*, Ruiz M, Pilon M.
Genetics 2018

IV. **Membrane Fluidity is Regulated by the *C. elegans* Transmembrane Protein FLD-1 and its Human Homologs TLCD1/2**
Ruiz M., Bodhicharla R., Svensk E., <u>Devkota R.</u>, Busayavalasa K., Palmgren H., Ståhlman M., Borén J., Pilon M.
Elife 2018

V. **Leveraging a Gain-of-Function Allele of *C. elegans* PAQR-1 to Elucidate Membrane Homeostasis by PAQR Proteins**
Busayavalasa K., Ruiz M., <u>Devkota R.</u>, Ståhlman M., Bodhicharla R., Svensk E., Hermansson N., Borén J., Pilon M.
PLoS Genetics 2020

VI. **Adipor2 is Essential for Membrane Lipid Homeostasis in Response to Dietary Saturated Fats**
<u>Devkota R.</u>*, Ruiz M.*, Palmgren H.*, Henricsson M., Jaiswal H., Maresca M., Bohlooly-Y M., Peng X-R, Borén J., Pilon M.
Manuscript

APPENDIX

I. **A Powerful Method to Evaluate Membrane Fluidity in *Caenorhabditis elegans*.**
<u>Devkota R.</u>, Pilon M.
BioProtocol 2018

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INTRODUCTION

This thesis begins with a brief overview of biological membranes, membrane homeostasis and regulation, and followed by a description of dedicated membrane sensors that act as sense-and-response proteins. Lipids are the primary constituent of membranes and, therefore, some key aspects of lipid metabolism are discussed in both *Caenorhabditis elegans*, the model organism most used throughout this work, and mammalian cells, where we have verified our fundamental findings. This will be followed by a section concerning glucose metabolism in *C. elegans* and finally a detailed overview of the adiponectin receptors, which are proteins of interest in this thesis and that belong to the PAQR family of proteins.

MEMBRANE HOMEOSTASIS

Introduction to the biological membranes

Biological membranes are vital for all cellular processes: they define cellular integrity, form the boundaries of organelles, act as cargo transporters (1), mediate vesicular trafficking (2), maintain ionic and osmotic gradients (3) and harbor transmembrane proteins that act as receptors, transporters or channels for critical biological function (4). Cellular membranes are primarily composed of a lipid bilayer, and recent advances in lipid analytics have shown that membranes contain a variety of different lipid species (5). Membrane lipids in eukaryotic cells have a remarkable structural diversity and the propensity of lipid variation influences the physical membrane properties such as thickness of the bilayer, density of lipid packing, fluidity and curvature (6–8). The major structural lipids in eukaryotic membranes are the glycerophospholipids, which are composed of a hydrophilic glycerol 3-phosphate-derived head group attached to two saturated or unsaturated acyl chains of varying lengths (Fig.1A-B).

The composition of acyl chain as well as the nature of the head group also influence the physical properties of the membrane (9). Phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) are zwitterionic in nature while phosphatidylserines (PSs) and phosphatidylinositols (PIs) are anionic (7). In addition, PCs are cylindrical-shaped phospholipids while PEs tend to form conical shaped phospholipids (10, 11), a physical property that influences membrane curvature and packing (12). Modulation of the relative abundance of PCs and PEs have also been shown to alter membrane fluidity (13), a membrane property that property that tends to correlate inversely with thickness, viscosity and packing, but directly with permeability of the lipid bilayer (Fig.1C-D).

The spectrum of acyl chains at the *sn1* and *sn2* positions in glycerophospholipids fall into three classes: saturated (e.g. palmitic acid (PA)/ C16:0), monounsaturated (e.g. oleic acid (OA)/C18:1) and polyunsaturated (e.g. arachidonic acid (AA)/C20:4 for the ω6 subclass, and eicosapentaenoic acid (EPA)/C20:5 for the ω3 subclass). Membrane rigidifying SFAs facilitate tight packing of the lipid bilayer and usually occupy the *sn1* position in a typical phospholipid. In contrast, the *sn2* position can be occupied by either a saturated or unsaturated fatty acid, and

increasing the degree of unsaturation (number of double bonds) in these UFAs leads to pronounced kinks in the acyl chain, which interferes with tight packing and therefore has an important influence on membrane properties such as fluidity (14, 15) (Fig. 1E-F).

Membrane fluidity, a recurring concept in this thesis, refers to the general mobility, i.e. rate of molecular motion, of the membrane lipids and proteins within a phospholipid bilayer. The fluidity of membranes is directly affected by temperature (16), degree of unsaturation in the acyl-chains (14) and also the presence of cholesterol in the membrane (17, 18). The influence of temperature and acyl chain unsaturation in membrane fluidity is particularly relevant for this thesis. With a drop in temperature, the phospholipid bilayer undergoes a reversible change from a fluid or disordered state to rigid or ordered state, resulting in a tightly packed bilayer with reduced membrane fluidity (19). As mentioned earlier, increasing the degree of unsaturation among acyl-chain introduces kinks in their structure and hence disrupts the order of the bilayer and thus promotes fluidity (14, 20).
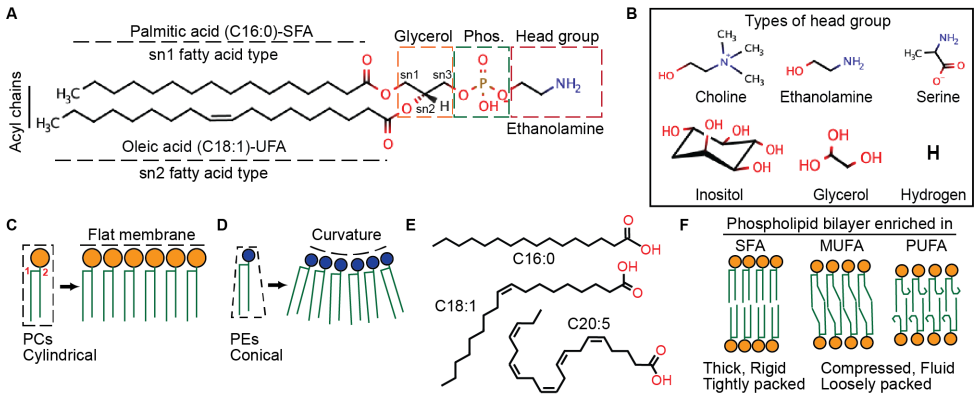
Fig.1. Overview of biological membranes. A) Structure of a typical phospholipid with an ethanolamine head group attached to a glycerol-3-phosphate backbone carrying fatty acyl chains at the *sn1* and *sn2* position. B) Diversity of head groups. C) Schematic representation of phosphatidylcholines, which tend to form a flat membrane because of their cylindrical nature. D) Cartoon representation of phosphatidylethanolamines which tend to form a curved membrane because of their conical nature. E) Diversity of acyl chains illustrated with three examples (many more variations exist): C16:0, palmitic acid is a SFA. C18:1, oleic acid is a monounsaturated fatty acid (MUFA); and C20:5, eicosapentaenoic acid, is a ω3 polyunsaturated fatty acid (PUFA). F) Phospholipid bilayers enriched in SFAs tend to be tightly packed and rigid while those enriched in MUFAs or PUFAs tend to be loosely packed, fluid and thinner.

Regulation of membrane homeostasis

Temperatures and Membranes. The first mechanistic insights into membrane homeostasis originate from the 1970s when a study reported that *Escherichia coli* adapt to

temperature by modulating their membrane composition in order to maintain membrane properties. Specifically, upon increasing cultivation temperatures, *E. coli* can incorporate increasing amounts of SFAs into membrane phospholipids to maintain constant membrane fluidity, a process termed "homeoviscous adaptation" (21). The physical properties of lipids in biological membranes are extremely sensitive to changes in temperature and hence homeoviscous adaptation has a paramount importance not only in bacteria (22), but also in all poikilothermic organisms that must counter temperature-induced perturbations in membrane organization (23). For example, fish respond to cold temperatures by increasing the production and incorporation of polyunsaturated fatty acids (PUFAs) into the membrane phospholipids and in some cases, by increasing the relative abundance of PEs (24, 25). Similarly, recent studies in *Drosophila melanogaster* have also shown that modulation of PUFAs levels is one of the fundamental mechanism contributing to cold adaptation in insects (26–28). The incorporation of PUFAs in membrane phospholipids lowers the transition temperature, i.e. the temperature at which the phospholipid bilayer undergoes a change from disordered to ordered state increasing rigidity, and hence counters cold-induced membrane rigidification (19).

The homeoviscous adaptation response has also been well documented in the nematode *Caenorhabditis elegans* (29). In the laboratory condition, *C. elegans* can easily be cultivated across a range of temperature spanning from 15 to 25 ºC (30). Worms induce the expression of the Δ9 FAT-7 desaturase upon transfer to cold temperature (31). The FAT-7 desaturase is the enzyme that inserts the first double bond between carbons 9 and 10 of a saturated stearic acid (SA, C18:0), resulting in the production of monounsaturated OA (C18:1n-9) (32). Unlike mammals, *C. elegans* has the ability to use 18:1n-9 as a substrate for further desaturation, elongation and *de novo* synthesis of PUFAs (33). Therefore, the relative abundance of OA in membrane phospholipids and neutral lipids in worms is relatively lower in comparison to that in mammalian membranes (29). The increase in PUFAs during cold adaptation has been previously investigated in *C. elegans* (31, 34). These studies have especially shown that the fraction of SFAs is markedly reduced in worms cultivated at 10 or 15 ºC compared to 20 or 25 ºC while PUFAs, such as linoleic acid (LA, 18:2n-6) and eicosapentaenoic acid (EPA, 20:5n-3), are increased in membrane phospholipids when worms are cultivated at lower temperatures. Further, genetic interaction studies with the three *C. elegans* genes encoding Δ9 desaturases, i.e. *fat-5, fat-6* and *fat-7,* have shown that Δ9 desaturation is critically important for survival at lower temperatures. Specifically, *fat-6;fat-7* double mutants show severely abnormal fatty acid composition (the mutants accumulate very high levels of 18:0, completely lack OA and other PUFAs derived from OA) accompanied by reduced survival at 10 or 15 ºC (35); the *fat-5;fat-6;fat-7* triple mutant is dead at all temperatures (36). Another mutant that is intolerant of cultivation at 15 ºC is the *paqr-2* mutant. *paqr-2,* encodes an adiponectin receptor homolog*,* and the mutant has reduced expression of the *fat-7* desaturase (37, 38). Conversely, the resilience of the cold-tolerant phosphoinositide-3 kinase *age-1* mutant is dependent on the activity of Δ9 desaturation (39). At the other end of the temperature spectrum, it has been suggested that acyl-CoA dehydratase ACDH-11-mediated reduction of fatty acid desaturation is important so that more SFAs can be incorporated into membrane phospholipids in response to increased temperature (40).

Membrane heterogeneity within eukaryotic cells. Temperature is not the only factor that influences the level of unsaturation in biological membranes. Intracellular regulatory mechanisms clearly exist that influence the huge diversity in lipid composition among the various organelles of eukaryotic cells, including in homeothermic mammals (41). It has been established that a typical eukaryotic cell exhibits a lipid gradient with sphingolipids, sterols and saturated acyl chains being gradually enriched along the membrane synthesis/secretory pathway ranging from higher fluidity and permeability in the endoplasmic reticulum (ER) membrane to increased rigidity and impermeability in the plasma membrane (20, 42). Sterols are almost absent in the ER but abundant in the trans-golgi and plasma membrane. Like sterols, sphingolipids accumulate in the plasma membrane but are rare in the ER (6, 7). This gradient results in increased membrane thickness across the secretory pathway which further influences protein localization in different organelles since the length of the transmembrane domains also varies among membrane proteins. Consequently, it has been suggested that one criterion for sorting membrane proteins to their site of action is to fit the length of their transmembrane domain to the thickness of the bilayer (41). Mammalian cells are also capable of homeoviscous adaptation, as first reported in 1980s when Chinese Hamster Ovary (CHO) cells, defective in the regulation of cholesterol biosynthesis were shown to accumulate excess cholesterol in their membranes compared to wild-type cells but still maintained normal membrane fluidity due to a compensatory threefold higher accumulation of OA (43).

Diets and membrane lipids. It has been well documented yet under-appreciated that mammalian membrane homeostasis is directly challenged by dietary lipid variation (44–47). From butter that is rich in saturated fats to olive oil containing up to 77% of OA and fish oil that is highly enriched in ω-3 PUFAs, dietary lipid variation is enormous. The incorporation of dietary fatty acids into mammalian membranes has been known for decades (44, 48–50). A number of studies in rats have shown that membrane fatty acid composition, especially the relative levels of ω-3 and ω-6 PUFAs, is highly influenced by changes in the dietary fat profile. However, the relative levels of SFAs and MUFAs in membrane phospholipids are highly regulated and remain relatively constant irrespective of large variation in the diet (47, 51). On the other hand, triacylglycerides (TAGs) in the adipose tissue are highly responsive and closely match the composition of the dietary fat (47). Further, analysis of fatty acid composition of serum phospholipids in postmenopausal Canadian women have shown a direct correlation between ω-3 PUFA supplementation and ω-3 PUFA incorporation in membrane phospholipids (48).

Membranes and energy costs. The intriguing "membrane pacemaker theory" of metabolism suggest that membranes play a fundamental role in determining the metabolic activity of any organism or tissue, and that this rate is highly influenced by the composition of the cellular membranes (52, 53). In particular, the membranes of metabolically highly active systems/tissues contain more polyunsaturated fatty acids (ω-3 PUFAs) (53). Additionally, membrane-influenced cellular processes are highly energy demanding (53, 54). For example, a study in rat thymocytes found that a substantial portion of the cellular energy is consumed by membrane-related processes that are strongly affected by the acyl composition of the membrane. Specifically, proton leakage accounts for 39% of the total basal metabolic rate while sodium pumping accounts for 10%, calcium pumping 10%, protein synthesis 20%, nucleotide

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synthesis 15% and the remaining 6% is consumed by other unidentified ATP consuming processes. To emphasize: it is astonishing that nearly 60% of a cell's energy is used for the maintenance of ion gradients across the membrane bilayer (53), a process strongly affected by the membrane composition (51). Additionally, membrane homeostasis itself is costly. For example, in the nematode *C. elegans,* almost 70% of the membrane phospholipids are replaced daily (55) and such a turnover rate, required for membrane homeostasis, must come at a significant energy cost.

Membrane property sensors

Recently, a number of sense-and-response proteins have been identified that maintain active and tight regulation of membrane properties. The following sections will present a brief synopsis of some of the best-understood membrane property sensors (further summarized in Table 1).

DesK: thermosensor in *Bacillus subtilis*

DesK is a transmembrane histidine kinase, initially identified as the sensor protein of a two-component system dedicated to maintain membrane fluidity at low temperatures in *Bacillus subtilis* (56). Following a drop in temperature, the DesK membrane spanning domain senses an increase in bilayer thickness due to increased ordering among the fatty acyl chains. This leads to stabilization of the kinase-dominant conformation of DesK, which causes autophosphorylation and then transfer of the phosphate group to DesR (DNA-binding response regulator). Phosphorylation activates DesR that then initiates the transcription of the Δ5 desaturase *des* gene followed by desaturation of the saturated acyl chain in membrane phospholipids, hence increased membrane fluidity. The resulting fluid membrane is also thinner since the disordered UFAs can again interdigitate, which switches DesK from its kinase-dominant to the phosphatase-dominant conformation, leading to dephosphorylation of DesR and turning-off of the transcription of the *des* gene (19, 57).

MGA2: sensing lipid saturation in *Saccharomyces cerevisiae*

The genome of *Saccharomyces cerevisiae* encodes a single and essential Δ9 fatty acid desaturase, OLE1, regulated by MGA2, a membrane bound transcription factor in the ER (58). It has been shown that Mga2 acts as a homo-dimer and uses a rotation-based mechanism for membrane sensing to regulate fatty acid desaturation. Specifically, this mechanism of membrane property sensing relies on the interaction of a bulky tryptophan residue (W1042) with the lipid environment. Disordered lipid packing stabilizes the transmembrane Mga2 helix dimer at a confirmation where the two tryptophan residues, located in the deep hydrophobic core of the membrane are facing away from each other and are easily accommodated by the membrane lipids. As fatty acid saturation increases within membrane phospholipids, the bulky W1042s are forced inward, stabilizing an alternative rotational orientation of the transmembrane helix dimer, where the W1042s are packed away from the lipid environment.

This structural dynamics of the transmembrane helix is proposed to regulate ubiquitylation and activation of MGA2 and, therefore, of the downstream OLE pathway (59).

OPI1 Sensing Phosphatidic Acid in *Saccharomyces cerevisiae*

Opi1 is a transcriptional repressor in *S. cerevisiae* that controls the expression of lipid biosynthetic genes involved in the production of membrane phospholipids such as PCs, PEs, phosphatidylinositol (PI) and phosphatidylserine (PS), for all of which, phosphatidic acid is the major precursor. When phosphatidic acid levels are high, Opi1 binds to phosphatidic acid via its amphipathic helix and is retained in the ER. As a result, Opi1 is prevented from entering the nucleus, thereby allowing the expression of the genes involved in the synthesis of membrane phospholipids. When phosphatidic acid is consumed, Opi1 is released and translocates to the nucleus where it represses the transcription of membrane lipid biosynthetic genes. Mechanistically, Opi1 uses a single amphipathic helix to sense both phosphatidic content as well as lipid packing in the ER (60).

SREBP1/SREBP2: sensing cholesterol and phosphatidylcholine

The Sterol Response Element Binding Proteins (SREBPs) are possibly the best understood sense-and-response proteins that act as both as lipid composition sensors and transcription factors to maintain cellular cholesterol and lipid homeostasis (61, 62). While SREBP2 senses cholesterol levels and regulates cholesterol biosynthesis, SREBP1 (isoforms 1a and 1c) primarily regulates lipogenesis and fatty acid desaturation in response to change in PC levels (63, 64). SREBP2 in the ER forms a heterodimeric complex with SREBP cleavage-activating protein (SCAP), which interacts with insulin-induced gene protein (INSIG), an ER-retention protein that retains the SREBP-SCAP complex on the ER membrane (62). As the cholesterol level decreases, INSIG becomes ubiquitylated and degraded which allows SREBP-SCAP complex to be transported from the ER to the Golgi apparatus via COPII vesicles. In the Golgi, the SREBP-SCAP complex is retained by the seven transmembrane protein PAQR-3 (65), and cleaved by membrane bound peptidases site-1 and site-2 proteases, resulting in the release of N-terminal domain that enters the nucleus and facilitates the transcription of genes involved in cholesterol and lipid synthesis (62). The evolutionary conserved SREBP1/PC feedback circuit involves Arf1-dependent processes (66) and the evidence suggests that SREBP1 activation is highly influenced by levels of PUFAs (67, 68). A study in mice has shown that dietary PUFAs dramatically decrease the levels of the active N-terminal mature form of the SREBP1 protein, accompanied by downregulation of lipogenic genes. However, PUFAs have no effect on either the mRNA or membrane-bound precursor SREBP1 protein levels, suggesting a posttranslational regulation (67).

PCYT1A: sensing membrane packing defects

The CDP-choline pathway, which corresponds to one half of the "Kennedy pathway", is essentially the sole *de novo* pathway for PC synthesis in almost all eukaryotic cell types. In this pathway, the enzyme PCYT1A (phosphate cytidyltransferase 1), aka CCT (choline

phosphate cytidyltransferase), catalyzes the formation of CDP-choline, which is the rate limiting step. Mammalian PCYT1A/B and yeast Pct1 are known to be amphitropic enzymes and are active when bound to the membranes (69). It has been suggested that the membrane binding of PCYT1A occurs via its amphipathic helix and that the catalytic efficiency increases by about 50 fold when membrane bound (70). Recently, it has been shown that during PC deficiency, membrane lipid stored curvature elastic stress (SCE) in the nuclear membrane increases, which allows PCYT1A to interact with the nuclear membrane, leading to its activation and thus increased synthesis of CDP-choline that ultimately lead to restoration of PC levels. Once PC levels are restored to alleviate SCE and maintain membrane packing, membrane binding by the PCYT1A amphipathic helix is disrupted, leading to its inactivation (71).

IRE1: sensing unfolded protein and ER stress

Accumulation of misfolded proteins in the membrane and lumen of the ER leads to a condition referred to as ER stress. The cellular machinery promptly responds to this stress via the endoplasmic reticulum unfolded protein response (ER-UPR), an evolutionary conserved stress response program that is present in perhaps in all eukaryotic cells. Inositol-requiring enzyme 1 (IRE1), a single-pass ER membrane protein, with a kinase and RNase domain, is fundamental for the activation of the ER-UPR. Unfolded proteins in the ER cause the oligomerization of IRE1, leading to autophosphorylation of the kinase domain and activation of the RNase domain. The RNase domain mediates an alternative splicing of the XBP1 transcript such that it then encodes a transcription factor that stimulates transcription of hundreds of ER response genes (72, 73). It has also been reported that a variety of membrane alterations, including high PC to PE ratio, increased lipid saturation, increased sterol levels and inositol depletion also activates IRE1-dependent ER-UPR (73). Recently, in *S. cerevisiae,* a mechanism for IRE1 activation by lipid bilayer stress has been described. The transmembrane domain of IRE1 consists of an amphipathic helix, that is juxtaposed with the membrane, and a short transmembrane helix that is perpendicular to the amphipathic helix. The transmembrane domain is anchored by polar residues in the cytosolic side and the amphipathic helix on the ER-lumen side. This creates a local membrane compression that will drive the clustering of IRE1 because clustering reduces the total area of membrane that must be compressed and thus reduces the thermodynamic energy cost. Lipid compositions that lead to more ordered and thicker membrane bilayer resists the compression, increasing its energy cost, thus leading to increased clustering and oligomerization of IRE1(74).

Membrane Sensors	What do they sense? (Cause)	Function (Effect)
DesK (**Bacteria**) in Plasma Membrane	Increased ordering and thickness of the phospholipid bilayer switches confirmation from phosphatase to kinase	Activates fatty acid desaturation
MGA2 (**Yeast**) in ER	Increasing membrane order promotes the rotation of MGA2 transmembrane helix that results in dimerization	Activates fatty acid desaturation
OPI1 (**Yeast**) in ER	Phosphatidic acid levels. Binds to phosphatidic acid when levels are high. Low levels promote translocation from ER to nuclei	Represses synthesis of other phospholipids, restores phosphatidic acid levels
SREBPs (**Eukaryotes**) in ER	Cholesterol levels. Lower levels lead to translocation from ER to Golgi and further cleavage of active N-terminal domain that translocate to the nucleus	SREBP1: Promotes *de novo* lipogenesis SREBP2: Promotes cholesterol synthesis
PCYT1A (**Eukaryotes**) in inner nuclear membrane	Membrane curvature stress. PC deficiency leads to increase in membrane curvature stress which is sensed by PCYT1A via its amphipathic helix	Interacts with the membrane and promotes PC synthesis
IRE1(**Eukaryotes**) in ER	Increased membrane thickness leads to clustering and oligomerization of IRE1	ER-UPR activation

Table 1. Brief synopsis of essential facts concerning membrane property sensors.

LIPID METABOLISM

The following sections form a short synopsis of lipid and fatty acid metabolism in *C. elegans* and mammalian cells, relevant for this thesis.

Lipid metabolism in *C. elegans*

Over the last few decades, the nematode *C. elegans* has been extensively studied to better understand the regulation of fatty acid and lipid metabolism. Lipid research in *C. elegans* so far has been focused on characterizing lipid biosynthetic genes, genes that regulate lipid storage and breakdown, as well as to understand the consequences of mis-regulation in lipid homeostasis. The worm genome contains at least 471 lipid metabolism genes (75) including genes involved in biosynthesis of PUFAs (76), mono-methyl branched fatty acids (77), phospholipids, sphingolipids (78) and TAGs (79).

Fatty acid synthesis and metabolism

Fatty acids in *C. elegans* lipids are typically 14-20 carbons long (29, 34). Fatty acids, which are the main component of neutral storage lipids, membrane lipids and signaling lipids can be saturated, with no double bonds, monounsaturated, with one C-C double bond, or polyunsaturated, with two or more C-C double bonds. Fatty acids are either *de novo* synthesized and/or obtained from the dietary *E. coli* OP50. Specifically, 7-20% of fatty acids are synthesized *de novo* from acetyl-CoA, while the remaining fatty acids are incorporated or modified from dietary fatty acids (80). This reflects the huge impact of bacterial diets on the *C.elegans* lipidome (81). Exception to this are the mono-methyl branched chain fatty acids, which are exclusively synthesized by the nematode (77, 80) and SA, OA and linoleic acid (LA, C18:2), synthesized through modification of PA (76, 80). A study using ^{13}C and ^{15}N stable isotopes-enriched diets have reported that the synthesis and turnover of fatty acids in *C. elegans* is rapid, where 4.5% of membrane fatty acids and 2.7% of storage lipids are turned over every hour. In fact, and almost 70% of the membrane is renewed every day (55).

The rate-limiting step in the *de novo* synthesis of fatty acids from two-carbon acetyl-CoA is the generation of malonyl-CoA, which is the product of acetyl-CoA carboxylase (ACC), i.e. *pod-2* in *C. elegans*. Malonyl-CoA is further acted on by the enzyme fatty acid synthase (FAS), for the synthesis of PA (Fig.2A). Hypomorphic mutants for these two rate-limiting enzymes *pod-2* and *fasn-1* arrest in early larval stages, implying that fat synthesis is essential for normal development (82). PA can be incorporated into TAGs or phospholipids or can be modified by the ELO-1, ELO-2 and ELO-3 elongases, the ART-1 enoyl reductase, LET-767 and the HPO-8 dehydratase and seven different desaturases (FAT-1 through FAT-7) to generate a variety of long chain PUFAs (LCPUFAs) (29). The amount of SFAs are relatively low in the worm lipidome with approximately 5% of fatty acids in total lipid extracts being 16:0, 6% 18:0, and less than 2% of 14:0 and 20:0 (34, 76). Conversely, MUFAs levels are quite abundant especially cis-vaccenic acid (18:1n-7), which is directly obtained from the *E.coli* OP50 (76). MUFAs are also produced from SFAs by desaturation by the Δ9 desaturases FAT-5, FAT-6

and FAT-7. The FAT-5 desaturase specifically acts on PA, producing palmitoleic acid (16:1n-7), while the FAT-6 and FAT-7 desaturases act on SA, producing OA (32) (Fig.2A).

Unlike mammalian cells, *C. elegans* possesses the ability to *de novo* synthesize a wide range of PUFAs. The $\Delta12$ desaturase FAT-2 converts OA into LA and FAT-1 converts omega-6 fatty acids into omega-3 fatty acids (83). C18 and C20 PUFAs constitute 28% of total fatty acids in total worm. EPA, the final product of fatty acid desaturation is the most abundant PUFA in all *C. elegans* lipid classes (29) (Fig. 2A). Even though PUFAs are required for growth, reproduction and neurotransmission (76), desaturases mutants important for PUFA biosynthesis are still viable, but with many developmental phenotypes (84, 85). $\Delta12$ desaturation (conversion of 18:1n-9 to 18:2n-6) is the critical step in PUFA biosynthesis in *C. elegans* and $\Delta12$ desaturase *fat-2* mutant completely lacks all normal PUFAs. However, these mutants accumulate different PUFAs species that are normally not detectable in worms (76), and this compensation probably explains the viability of these mutants, though they still display severe developmental defects.
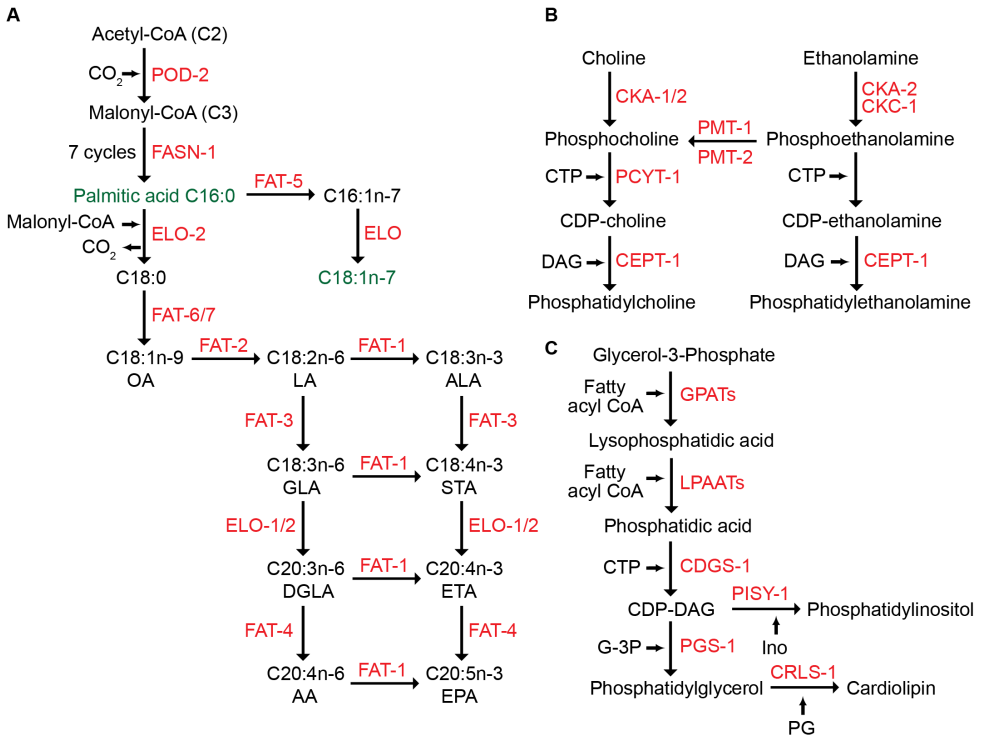
Fig. 2. Overview of the fatty acid and phospholipid synthesis pathways in *C. elegans*. A) Schematics showing the fatty acid synthesis pathway in *C. elegans*. Enzymes involved are labeled in red. Fatty acids in green can also be readily obtained from the diet. (Adapted from (29)). B) Kennedy pathway in *C. elegans* for the synthesis of phosphatidylcholines and phosphatidylethanolamines. Enzymes involved are labeled in red. (Adapted from (64, 86)). C) Phosphatidic acid synthesis and CDP-DAG pathway. (Adapted from (29)).

Fatty acid catabolism occurs via the process of β-oxidation by which a cascade of enzymes coordinately cleaves the C-C bonds to produce energy. β-oxidation in *C.elegans* occurs in two major metabolic organelles, the mitochondria and peroxisomes (29). The relative proportions of fatty acid oxidation that takes place in mitochondria versus peroxisomes is not clearly known for *C.elegans,* but genetic screens have suggested that peroxisomal oxidation plays a particularly important role in the breakdown of fats from large lipid droplets (87, 88). Further, it has been shown that inhibiting the rate limiting enzymes in β-oxidation lead to fat accumulation in lipid droplets, a form of neutral lipid storage (89).

Intestinal and hypodermal cells are the major sites of fat storage in *C elegans*. The intestine is an important tissue for lipid synthesis, uptake, storage and mobilization, but different lipid biosynthetic and degradation enzymes are also expressed in hypodermis (90). *de novo* synthesized or diet-acquired fatty acids are first converted into TAGs, which are the major component of lipid droplets (LDs) that accumulates in intestine, hypodermis and germline. The core of the LDs consists primarily of TAGs which is enclosed within a phospholipid monolayer (91, 92). Intestinal cells also synthesize and transport vitellogenins, a yolk protein equivalent to mammalian lipoproteins, to the developing oocytes. Vitellogenins are secreted from the intestine into the pseudocoelom and are eventually endocytosed through the RME-2 receptor in the maturing oocytes via a process called receptor-mediated endocytosis (93). It has been shown that the lipids associated with purified yolk complexes from the adult hermaphrodite consists of PCs, PEs, TAGs and free fatty acids (94).

Biosynthesis of phospholipids

Phospholipids are the primary constituent of *C. elegans* membrane, with PCs and PEs being the most abundant phospholipid class, followed by PIs or PSs (55, 95). *C. elegans* uses both the cytidine diphosphate diacylglycerol (CDP-DAG) pathway and the Kennedy pathway for the synthesis of phospholipids. In the Kennedy pathway, choline and ethanolamine are first phosphorylated by choline and ethanolamine kinases (CKA-1, CKA-2, CKB-1, CKB-2, CKB-3, CKB-4) to form phosphocholine and phosphoethanolamine, then activated by CTP-phosphocholine and CTP-phosphoethanolamine cytidylyl transferase (PCYT-1) to form CDP-choline and CDP-ethanolamine, which further react with DAG to form PC and PE respectively. Additionally, PEs can further be converted into PCs by phosphoethanolamine methyl transferase enzymes PMT-1 and PMT-2 (Fig.2B). Preceding the CDP-DAG pathway, acyl groups are first converted to acyl-CoAs by acyl CoA synthetase (ACS). Then, acyl-transferase (GPAT and LPAAT)-mediated addition of two acyl-CoA groups to glycerol-3 phosphate (G3P) leads to the synthesis of phosphatidic acid, a major metabolite in the synthesis of both neutral lipids and phospholipids. In the CDP-DAG pathway, phosphatidic acid and CTP are combined, leading to the formation of CDP-DAG, and ultimately to the synthesis of PIs and cardiolipin (CL), a major lipid moiety in mitochondrial membranes (29) (Fig. 2C).

Regulation of Δ9 desaturation

As mentioned earlier, the genome of *C. elegans* encodes three Δ9 desaturases: *fat-5* which converts C16:0 to C16:1, and *fat-6/7* which act on C18:0 to generate C18:1. The process of Δ9 desaturation is extremely important since it is the sole pathway that provides precursors for PUFA synthesis. Several studies, mostly in mammalian cells, have shown that the levels of Δ9 desaturase activity are primarily regulated at the level of transcription because both the protein and the mRNA have short half-lives (96–98). In *C. elegans*, a number of studies have reported on the mechanisms by which transcriptional regulation of Δ9 desaturases is achieved in response to changes in the environment or diet. Notably, expression of the *fat-7* desaturase is highly influenced by changes in ambient temperature or food deprivation: worms shifted to a lower temperature strongly upregulate the expression of *fat-7* (31), while worms deprived of food markedly reduce its expression (99). A number of transcription factors regulate the expression of *fat-7*. These include the nuclear hormone receptors *nhr-49* (homologous to mammalian PPARα/HNF4α) (100), *nhr-80* (36) and *nhr-13* (101); both *nhr-80* and *nhr-13* can act as co-factors of *nhr-49* to regulate Δ9 desaturases (101). *sbp-1*, a homolog of mammalian sterol regulatory element binding protein (SREBP) (102), and *mdt-15*, a homolog of mammalian MED15 (ARC105), that act as a mediator subunit and facilitates the activity of both *nhr-49* (103) and *sbp-1* (102), also regulate the expression of desaturases. Additionally, there are reports suggesting that heat shock factor 1 (*hsf-1*) and a FOXO-family transcription factor (*daf-16*) can also regulate the expression of *fat-7* (104, 105).

Lipid metabolism in mammalian cells

The lipid composition of a typical mammalian cell consists of 45-55% PCs, 15-25% PEs, 10-15% PIs, 5-10% PSs, 1-2% phosphatidic acid, 7-15% sphingomyelin, 2-5% cardiolipin, 2-5% glycosphingolipids, 10-20% cholesterol and <1% phosphatidylglycerol (106). Fatty acids, a major moiety of most of these lipids, are either obtained directly from the diet or synthesized *de novo* using acetyl-CoA and malonyl-CoA as the substrate. These fatty acids are acted on by acyl-CoA synthetases, then converted to acyl-CoAs and further enter into metabolic pathways including desaturation and elongation then channeled towards phospholipid or TAGs synthesis or degradation pathways (107). Fatty acid elongation occurs in the ER using malonyl-CoA as a carbon donor and catalyzed by ELOVLs. Fatty acid desaturation is carried out by stearoyl-CoA desaturase (SCD1 and SCD5 in humans), an ER resident enzyme that utilizes PA to synthesize MUFAs (108) (Fig. 3A).

There are two important differences between *C. elegans* and mammalian lipid metabolism which are especially relevant for this thesis. The most striking difference includes the synthesis of LA and alpha-linolenic acid (ALA, 18:3n-3), the essential fatty acids in mammals. In contrast to worms, mammalian cells are devoid of both Δ 12 and omega-3 desaturases and hence are not able to convert OA to LA and LA to ALA respectively. Therefore, mammalian cells must obtain these essential fatty acids from the diet (33). Once acquired, cells can elongate and desaturate these essential fatty acids to produce LCPUFAs including arachidonic acid (ARA, 20:4n-6), EPA and docosahexaenoic acid (DHA, 22:6n-3) with the

enzymes fatty acid desaturase 1 (FADS1) and fatty acid desaturase 2 (FADS2) (33, 109) (Fig. 3B-C). Cells can also obtain these LCPUFAs directly from the dietary source.

The role of cholesterol in membrane homeostasis is another aspect of mammalian lipid metabolism that is significantly different between mammals and *C. elegans*. Worms lack certain enzymes for cholesterol synthesis in the mevalonate pathway and hence are not able to *de novo* synthesize cholesterol (110). However, cholesterol is essential for growth, reproduction, survival and hormone production (111–114). In the laboratory, *C. elegans* are routinely grown in a culture medium supplemented with cholesterol, though the cholesterol levels in the cell membrane is very low (115, 116). The low amount of cholesterol required for a normal *C. elegans* development and health makes it unlikely that cholesterol plays an important structural role in cell membranes and their functions (112, 117). In contrast, mammalian cells can synthesize cholesterol in the ER from where it is rapidly transported to other organelles and gradually enriched in an increasing gradient along the secretory pathway (118). It has been estimated that the cholesterol content in the ER is as low as 1% of the total cell cholesterol. On the other hand, cholesterol accounts for 20-25% of the total lipids in the plasma membranes (119, 120). Cholesterol in mammalian membranes influences membrane fluidity and thickness which in turn affects the function of membrane proteins such as receptors, ion channels and transporters (121).

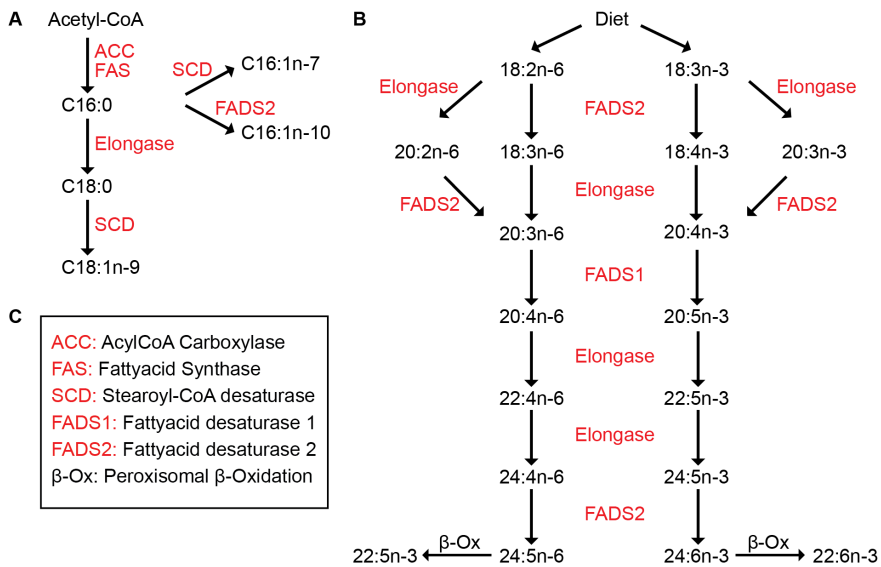**Fig. 3. Overview of monounsaturated fatty acid and polyunsaturated fatty acid synthesis pathways in mammalian cells.** A) Schematics of the monounsaturated fatty acid synthesis pathway in mammalian cells. B) The PUFA synthesis pathway in mammalian cells. Note that the essential fatty acids linoleic acid (18:2n-6) and alpha linolenic acid (18:3n-3, ALA) must be obtained from the diet. C) Names of the enzymes involved in the pathway.

GLUCOSE METABOLISM

The following section will provide a brief synopsis of glucose metabolism in *C. elegans* and provides a background with which to understand glucose toxicity, itself an important subject of this thesis.

Cellular glucose is either stored in the form of glycogen or metabolized via the glycolysis pathway (Fig. 4). Reducing glucose metabolism by inhibition of the glucose 6-phosphate isomerase (GPI-1) enzyme in the glycolysis pathway (122), or by inhibition of facilitated glucose transporter (FGT-1) (123), or using 2-deoxy-D-glucose to inhibit glycolysis (122), extends lifespan in *C. elegans*. Conversely, exogenous glucose supplementation in the culture plates has proved to be detrimental to the overall physiology of *C. elegans*. Concentrations of glucose ranging from 0.1% (5 mM) to 2% (111 mM) reduce the lifespan (122, 124–126), induce apoptosis (127), decrease brood size (128) and impair the response to oxygen deprivation (129). Increased glucose concentration also leads to the formation of methylglyoxal and advanced glycation end products (125, 130). Glucose-induced lifespan reduction is at least in part a result of downregulated DAF-16 (FOXO) and HSF-1 (heat shock transcription factor) activities and of their downstream target, the aquaporin glycerol channel (AQP-1). Further, glucose feeding increased glycerol levels and supplementing glycerol in the culture plates also decreased the lifespan. This suggests that the toxic effect of glucose might be through its conversion to glycerol (124) or that both glucose and glycerol metabolism lead to a common metabolite that is toxic.

In a separate study (126), feeding worms with glucose increased overall fat levels, TAGs and also the levels of SFAs and monounsaturated fatty acids (MUFAs). The authors suggested that the accumulation of SFAs might lead to the feedback inhibition of glucose metabolism to SFAs synthesis by blocking the upstream enzymes, which plausibly leads to accumulation of intermediate metabolites, some of which may be the toxic component of glucose feeding. In fact, feeding worms with dihydroxyacetonephosphate (DHAP), a glycolysis intermediary metabolite, significantly reduced the lifespan. Conversely, inhibition of fructose-1,6-bisphosphate aldolases (ALDO-1/2), the enzyme responsible for the production of DHAP, restored the lifespan on a glucose diet. Further, inhibition of SBP-1 and MDT-15 exacerbated the glucose-induced lifespan reduction, and the glucose-induced lifespan reduction could be partially rescued by UFA supplementation (126), likely because of their positive effects on membrane fluidity.

The question regarding whether the effect of glucose is directly on the worm or via the OP50 *E. coli* strain that is used as food source has been raised time and time again (122, 124, 129). Using *E. coli* mutants defective for glucose uptake or otherwise metabolically arrested, researchers have shown that the effect of glucose on longevity is a direct effect on the worm and not via bacteria (122, 124). However, the effect of glucose in response to oxygen deprivation has been shown to act through the OP50 dietary bacteria. The ability to survive upon exposure to 24 hours of anoxia was completely abolished when glucose was supplemented in the culture plates with *E. coli* OP50 but not with ΔPTS-OP50, a mutant strain unable to take up glucose (129). This clearly indicates that for certain phenotypes, understanding glucose metabolism in bacteria is critically important.
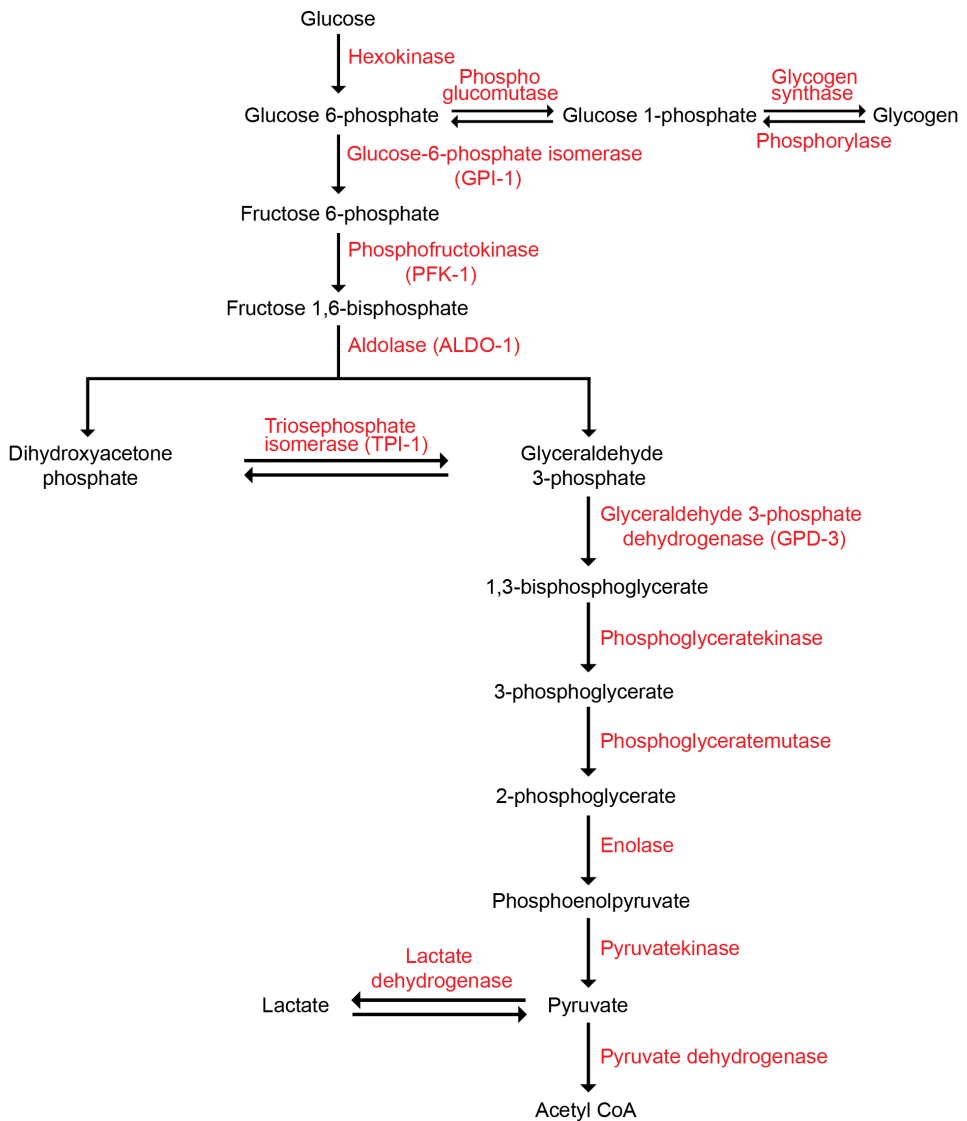
Fig. 4. Glucose metabolism and glycolysis pathway. A schematic representation depicting glucose metabolism in *C. elegans*. Glucose can be stored as glycogen or can be metabolized via the glycolysis pathway to generate pyruvate (adapted from (29, 131)). The enzymes involved in each step is labeled in red.

PAQR PROTEINS

A core subject of this thesis has been to understand the role of PAQR-2, a *C. elegans* homolog of mammalian adiponectin receptors, in membrane homeostasis. The following sections summarizes the current knowledge concerning the PAQR protein family, with a specific focus on adiponectin receptors and their homologs.

Overview of PAQR proteins

Members of the Progestin and AdipoQ Receptor (PAQR) protein family are well represented throughout the evolutionary tree: there is a single member in *E. coli*, two in *Bacillus cereus*, one in *Mycobacterium tuberculosis*, four in *S. cerevisiae*, four in *Saccharomyces pombe*, six in *Arabidopsis thaliana*, five in *C. elegans*, five in *D. melanogaster* and eleven members in *Mus musculus* and *Homo sapiens*. All PAQR proteins have a characteristic seven transmembrane domain with divergent intracellular N- and extracellular C-termini. PAQR proteins show no sequence homology with the well-known seven transmembrane G-protein coupled receptors (GPCRs) (132, 133). Mammalian PAQR-1 and PAQR-2 are suggested to act as adiponectin receptors, are better known as AdipoR1 and AdipoR2 respectively, and were initially identified in a screen for membrane proteins that bind adiponectin, a protein secreted by adipocytes (132). PAQR proteins have been studied in many of the above-mentioned model organisms, as will be briefly described in the following sections.

Yeast: PAQRs associated with a ceramidase activity

The genome of *S. cerevisiae* contains four members of the PAQR protein family. Expression of the yeast PAQR proteins, named as IZH1-4 (Implicated in Zinc Homeostasis) (134) is induced by increased zinc levels (134), inhibited by the presence of UFAs such as OA and LA and stimulated by the SFA myristic acid (MA, C14:0) and glucose (134–136). Specifically, the growth of the Izh2 mutant strain is hindered by myristate, glycerol or lactate. Mechanisms of resistance to nystatin, an antifungal compound known to bind to sterols in the membrane, in the Izh2 mutant have been speculated to be a result of altered membrane lipid composition (136). The IZH2 protein binds to osmotin, a plant protein secreted for defense mechanisms that shows some structural homology to adiponectin. Interestingly, osmotin is also able to induce the phosphorylation of AMPK in C2C12 myocytes and this phenomenon is dependent on AdipoR1/2 (137), suggesting that the structural homology with adiponectin allows it to act as activating ligands for the AdipoRs (137, 138). IZH2 negatively regulates the expression of *FET3*, a gene involved in iron uptake (139) and using this as a read-out reporter for IZH2 activity, a study published in 2009 showed that Izh2 act as a ceramidase, and that overexpression of IZH2 leads to the accumulation of sphingoid bases that can further activate the downstream signaling pathway (140). Further, D-erythro-MAPP, a known inhibitor of alkaline ceramidases was shown to inhibit IZH2 activity, which again implies that IZH2 possess a ceramidase activity (140, 141). To summarize, the yeast homologs of the mammalian AdipoRs possess an intrinsic ceramidase activity, are essential to challenges that disrupt

membrane homeostasis (SFA treatment, glucose treatment) and are transcriptionally inhibited in the presence of UFAs such as OA and LA.

Drosophila: PAQR associated with insulin secretion

There is a single AdipoR homolog in *Drosophila* (dADIPOR) and the null mutant is lethal (142), indicating an essential role. dAdipoR has 66% similarity with human AdipoR1 and is expressed throughout all developmental stages, and in the adult brain, including in insulin producing cells (IPCs), neurons, salivary gland, fat body and gut. IPCs-specific silencing of dAdipoR have resulted in several phenotypes including increased sugar level in the hemolymph, increased TAGs and reduced lifespan (143). *Drosophila* insulin-like peptides (Dilps) are produced by IPCs and a recent study showed that starvation induces the accumulation and prevents the secretion of Dilps. Upon feeding, the nutrient availability is sensed by the fat body, triggering the release of Dilps from IPCs (144). This process of insulin secretion was found to be altered by IPCs-specific dAdipoR inhibition, suggesting that dAdipoR plays a role in insulin secretion in flies (143). dAdipoR has also been associated with maintenance of germline stem cells in the *Drosophila* ovary (142). Keeping in mind the association of yeast IZHs with membrane homeostasis, the role of dAdipoR in insulin secretion in *Drosophila* is interesting. In particular, one could hypothesize that the insulin secretion defect observed in flies where dAdipoR is specifically silenced in IPCs is actually a result of a primary disruption of membrane homeostasis that secondarily affects insulin secretion.

C. elegans: PAQR proteins associated with regulation of fatty acid desaturation

The genome of *C. elegans* encodes five members of the PAQR family of proteins (37, 133), with PAQR-1 and PAQR-2 being most homologous to the mammalian AdipoR1 and AdipoR2 proteins (37). The *paqr-1*, *paqr-2* and *paqr-3* null mutants have been characterized in some details, and it was found that only the *paqr-2* mutant has a visible phenotype, namely a withered tail tip defect that is more pronounced in adults than in larvae. Additionally, the *paqr-2* mutant is cold intolerant (at 15°C), has a reduced lifespan, low brood-size and have abnormal locomotion. The *paqr-2* mutant has an altered fatty acid composition and is synthetic lethal when combined with loss-of-function (*lof*) mutations in some genes involved in lipid metabolism such as *nhr-49/*nuclear hormone receptor and *sbp-1* (the SREBP homolog mentioned earlier). The double mutant *paqr-1;paqr-2* has more aggravated phenotypes than either single mutants, suggesting functional redundancy between these two proteins. Expression of a *PAQR-2::GFP* translational reporter is consistently detected on the membranes of gonad sheath cells, and is also found in certain neurons in the head and the tail region (37). To better understand the role of *paqr-2* during cold adaptation in *C. elegans*, an unbiased forward genetic screen was performed to isolate mutants that suppress the inability of *paqr-2* to grow at 15°C (38). All of the isolated *paqr-2* suppressor mutants not only suppressed the inability of *paqr-2* to grow at 15°C but also suppressed the characteristic withered tail-tip phenotype, growth rate defect and the small brood size phenotype. The suppressor mutants were molecularly defined by whole-genome sequencing and can be broadly categorized into three pathways: a) genes

involved in the PC synthesis pathway (*lof* mutants *cept-1, pcyt-1* and *sams-1*) b) genes involved in fatty acid metabolism pathways (gain-of-function (*gof*) mutants *nhr-49, mdt-15* and *sbp-1*) c) genes involved in the fatty acid β-oxidation pathway (*lof* mutants *ech-7, hacd-1*). The *lof* mutants in the PC synthesis pathway as well as the *gof* mutants in fatty acid metabolism pathways could suppress the inability of *paqr-2* mutant to induce fatty acid desaturation. This is particularly interesting since an independent study reported that PC depletion results in the activation of *sbp-1*/SREBPs (64), suggesting that the induction of fatty acid desaturation by the *lof* mutants in PC synthesis pathway is via activation of *sbp-1*. Consistent with this hypothesis, lipidomics analyses have shown that *paqr-2* mutants accumulate excess SFAs in their membrane phospholipids (PCs and PEs), which is a direct consequence of the mutant's inability to induce the expression of FAT-7 desaturase and to convert SFAs to UFAs. In fact, supplementing the culture plates with OA, a product of FAT-7 activity, partially suppressed the cold intolerant phenotype and a combination of OA and low concentration of non-ionic detergents (e.g. NP40) completely suppressed the mutant phenotype. This is a striking result which suggest that the role of PAQR-2 is to promote fatty acid desaturation in order to maintain membrane fluidity during cold adaptation (38); homeoviscous adaptation is a fundamental cold adaptation mechanism in poikilotherms that has been known for decades but in which PAQR-type proteins were not previously implicated. To summarize, the adiponectin receptor homolog PAQR-2 in *C. elegans* regulates fatty acid desaturation and most likely acts via the transcription factors *nhr-49* or *sbp-1* since these transcription factors can either act themselves as *paqr-2* suppressors or likely act downstream of the other suppressor mutations.

Mammalian adiponectin receptors

The best studied members of the PAQR family of proteins are the mammalian adiponectin receptors. The following section will briefly describe the structure of the mammalian AdipoRs, their oligomerization and anterograde transport, phenotypic studies by different research groups in mouse AdipoR knockout models, and the AdipoR downstream signaling network that is known so far.

Crystal structure of the AdipoRs

The crystal structure of AdipoR1/2 (i.e. residues 89-375 for AdipoR1 and residues 100-386 for AdipoR2) without the N-terminal region was published in 2015, which confirmed the predicted seven transmembrane domains with cytoplasmic N-termini and extracellular C-termini typical of PAQR-type proteins (145). The structure of both AdipoR1 and AdipoR2 resembles a cylindrical barrel with a large internal cavity open towards the cytoplasm and that could allow the trafficking of substrates and products to and from an enzymatic site within the cavity. The presence of a zinc ion bound within the transmembrane barrel, coordinated by conserved histidine residues at H191, H337 and H341 in AdipoR1, and H202, H348 and H352 in AdipoR2, was predicted to play a role to stabilize the overall structure of the protein(145) (Fig. 5). In a separate independent study (146), other authors presented a revised AdipoR2 crystal structure and found the presence of free fatty acid molecule in the internal cavity of the

protein, which was further modelled to OA to fit within the density map (Fig. 5). Given the literature about yeast IZHs acting as a ceramidases (140), and mammalian AdipoRs possessing a ceramidase activity (141, 147), the authors have further confirmed biochemically that the AdipoRs definitely can act as ceramidases and that the activity can be further enhanced by adding adiponectin to an in vitro reaction. However, since the ceramidase activity was low, the authors do not rule out the possibility that the AdipoRs may have amidase activity for other natural lipids(146).

AdipoRs: Oligomerization and Anterograde Transport

The observation that the AdipoRs can form homo- and heteromultimers was published back in 2003 (132) and in a follow-up independent study (148), the occurrence of AdipoR1 dimers was reported in a number of cell lines, including HEK293, MCF-7, HUVEC as well as in human femoral muscle tissue. Using a Bimolecular Fluorescence Complementation (BiFC) assay in HEK293, the authors studied the dimerization of AdipoR1 in living cells and quantified the signal using flow cytometry. The dimerization was shown to be dependent on a GxxxG dimerization motif present in the fifth transmembrane domain (Fig. 5); replacement of the glycine residues to aromatic phenylalanine residue in this motif significantly increased the dimerization while substitutions to polar glutamic acid nearly abolished the dimerization (148). Homo- and heterodimers of AdipoR1 and AdipoR2 form with similar efficiency on the plasma membrane and also in the ER of HEK293AD cells (149).

A systematic N-terminal deletion approach was used to identify specific sequence motifs required for the export of AdipoR1 from the ER. In particular, two specific sequence motifs, $^{121}F(X)_3F(X)_3F^{129}$ and $^{106}D(X)_3L^{110}L^{111}$, proximal to the first transmembrane domain, mediates the exit of AdipoR1 from the ER (150) (Fig. 5). It has been shown both in Hela cells and HEK cells that the subcellular localization slightly differs between AdipoR1 and AdipoR2. The majority of AdipoR1 protein localizes to the plasma membrane while the bulk of AdipoR2 appears associated with the ER (151, 152). The non-conserved N-terminal residues (1-81) in AdipoR2 have been reported to restrict the plasma membrane localization of AdipoR2. This is supported from the use of chimera constructs, swapping the non-conserved regions to generate AdipoR1$_{(1-70)}$/R2$_{(82-386)}$-HA and AdipoR2$_{(1-81)}$/R1$_{(71-375)}$-HA: combining the AdipoR2 transmembrane domains with the AdipoR1 N-terminal region produces a chimeric protein that is readily detected on the plasma membrane, which is not the case with the complementary construct. Complete removal of the non-conserved N-terminal residues instead allowed the detection of both AdipoR1 and AdipoR2 at the cell surface, which further confirms that the AdipoR2 N-terminal region restricts its expression at the cell surface. Further, it was confirmed that AdipoR1 and AdipoR2 form heterodimers when co-expressed and that this also facilitated the plasma membrane expression of AdipoR2, suggesting that possibly the bulk of AdipoR2 at the cell surface might exist in the form of heterodimers (152). To summarize, the non-conserved N-terminal regions of AdipoR1 and AdipoR2 regulate their differential subcellular expression and the specific sequence motifs present in the higher homology region proximal to the first transmembrane domain mediates anterograde transport from the ER.
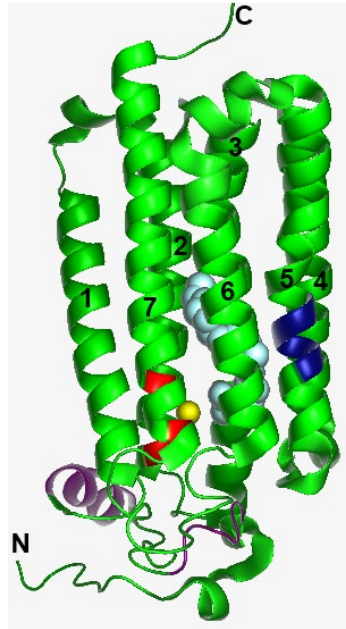
Fig. 5. 3D structure of crystallized AdipoR2. Structure of crystallized AdipoR2 (145, 146) depicting seven transmembrane domains (labeled from 1-7), with an intracellular N-terminus (N-terminal residues from 100-145) and an extracellular C-terminus. The presence of Zn atom (in yellow) was noticed in the AdipoR2 crystals along with a free fatty acid molecule which was later modelled to oleic acid (depicted using a pale blue ball and sphere model inside the cavity) to fit the electron density. The Zn-coordinating conserved histidine residues in transmembrane (TM) domains 2 and 7 are highlighted in red in the TM helix. The dimerization motif in TM 5 (148) is highlighted in dark blue and highlighted in purple are the N-terminal motifs proximal to TM 1, which are important for anterograde transport and localization to the plasma membrane (150). The AdipoR2 structure information was obtained from protein data bank (5LXA.pdb) and modified using the Pymol software.

Phenotypic study in mouse knockouts

Mouse and human AdipoR1 are 96.8% identical in their amino acid sequence while AdipoR2 is 95.2% identical between these two organisms. Mouse AdipoR1 is ubiquitously expressed, with skeletal muscle being the predominant site, while AdipoR2 is mostly expressed in the liver, though lower levels are found in many other tissues. Similar expression pattern has also been observed for the human proteins: human AdipoR2 is mostly expressed in the skeletal muscle and the liver while AdipoR1 is strongly expressed in most tissues, including skeletal muscle, heart, placenta, kidney and liver (132). Studies of AdipoR knockout mice from two independent labs were published in 2007, which reported rather different phenotypic observations (153, 154).

AdipoR1 single knockout mice (excision of exon 2, 3 and 4) from the Kadowaki lab developed normally and were fertile with a normal body weight. However, in an oral glucose tolerance test, these AdipoR1 knockout mice had increased plasma glucose and insulin levels compared to wild-type, suggesting insulin resistance. Endogenous glucose production was also increased in the AdipoR1 knockout mice. AdipoR2 single knockout mice (excision of exon 3) expressed low levels of an aberrantly spliced transcript that likely did not translate into any protein, even though protein expression was not thoroughly tested. These AdipoR2 knockout mice were also viable and fertile with no change in body weight. Unlike the AdipoR1 knockout mice, the AdipoR2 knockout mice did not show any glucose intolerance phenotype but their plasma insulin levels were significantly higher than in the wild-type mice. Endogenous glucose production in AdipoR2 knockouts was not different than wild-type. Importantly, the double AdipoR1 and AdipoR2 knockout mice were also viable and fertile with a normal body weight. However, they displayed increased insulin resistance compared to the single knockouts, as well as increased hepatic glucose production (153).

In the other study, the knockout AdipoR1 mice (excision of exon 2) and AdipoR2 mice (excision of exon 5) generated by Deltagen (San Carlos, CA) displayed contrasting phenotypes (154). These AdipoR1 knockout mice were obese (increased fat mass), which the authors correlated with decrease energy expenditure and decreased locomotion activity. Knockout males were glucose intolerant but had a normal insulin response. Females, on the other hand, had increased fasting glucose levels with unchanged insulin levels (154). Conversely, AdipoR2 knockout mice were lean and resistant to high fat diet-mediated weight gain, obesity and hepatic steatosis. Intriguingly, on a high fat diet, these AdipoR2 knockout mice had improved glucose tolerance and low levels of plasma glucose and insulin (154, 155). However, prolonged high fat diet for up to 20 weeks dramatically increased the levels of plasma glucose levels in these AdipoR2 knockout mice although the levels of insulin was unaltered, suggesting that pancreatic beta cells in the knockout are unable to compensate for the insulin resistance (155). The lean phenotype of AdipoR2 knockout mice was further correlated with increased energy expenditure and locomotion activity (154). Interestingly, the double knockout for these alleles of AdipoR1 and AdipoR2, constructed by Deltagen, was embryonic lethal and the embryos died before embryonic day 16.5 (156).

To summarize, the most important discrepancy between the knockout mice from the Kadowaki lab and those from Deltagen is the embryonic lethality found only in the double knockout generated from Deltagen. Further, the AdipoR1 and AdipoR2 single knockouts from Kadowaki lab showed similar trends in terms of insulin sensitivity and glucose tolerance while the Deltagen single AdipoR1 and AdipoR2 knockouts had opposing effects. These important discrepancies in the results obtained using two different sets of knockout mice makes it difficult to understand the true physiological roles of the AdipoRs.

The AdipoRs Signaling Network

Signaling downstream of AdipoR1/2 may occur through two key players: AMPK (AMP-activated protein Kinase) and PPARα (Peroxisome Proliferator Activated Receptor α) (153, 157–159). Under low-energy conditions, AMPK, also known as a guardian of

metabolism, promotes ATP synthesis and decreases ATP consumption through phosphorylation of a number of proteins in different pathways including lipid homeostasis, glycolysis and mitochondrial homeostasis. PPARα, one of three members of a small nuclear receptor family that also includes PPARγ and PPARδ, is a ligand-activated transcription factor that regulates fatty acid metabolism, including fatty acid transport and β-oxidation, as well as hepatic glucose production (160, 161).

The beneficial effects of AdipoR1 *in vivo* in mouse liver and muscle and also in cultured hepatocytes, appear dependent on the activation of AMPK (153, 158). On the other hand, the protective role of AdipoR2 *in vivo* in mouse liver appears entirely due to the activation of PPARα and its target genes, though not via AMPK (153), while in muscle, AdipoR2 does activate AMPK (162). AdipoR1/2 have also been shown to act as ceramidases, i.e. able to convert ceramides to sphingosine which can then be converted to the signaling sphingosine-1-phosphate (S1P), a signaling molecule (163), which protects cells from palmitate-induced cell death (146, 147). This particular AdipoR signaling mechanism is not dependent on AMPK, though the authors have suggested that AMPK could be a downstream target of S1P. (147). A cautionary note is in order here because this overview of the AdipoR signaling network is a compilation from studies using two different knockout mouse models that, as mentioned earlier, have shown contrasting phenotypes. In particular, the lethality of the Deltagen AdipoR1/2 double knockout mice likely explain why the authors studying the in vivo role of the AdipoR ceramidase activity resorted to investigating cultured mouse embryonic fibroblasts (MEFs) in order to measure ceramide levels (147). Discrepancies notwithstanding, one may still try to summarize the literature on AdipoR signaling in a succinct manner, as shown in Fig. 6.
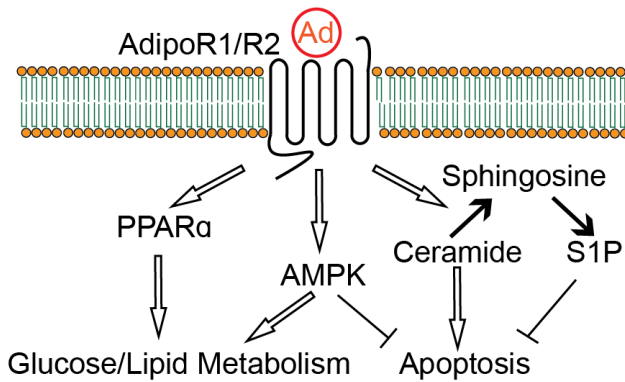
Fig. 6. Simplified proposed signaling pathways downstream of AdipoRs. According to published studies**,** binding of adiponectin activates AdipoR1/R2 which in turn activates AMPK and PPARα, which are involved in the regulation of glucose and lipid metabolism. AMPK also inhibit apoptosis. Additionally, AdipoR1/R2 can act as ceramidases, cleaving ceramides (induces apoptosis) to form sphingosine, which is readily phosphorylated to sphingosine-1-phosphate (S1P). S1P is an anti-apoptotic metabolite that protects cells from palmitate-induced cell death (Adapted from (147, 159)).

AIM

As the authors mentioned in 2013, 'PAQR-2 regulates fatty acid desaturation during cold adaptation in *C. elegans*' (38). This thesis is based on the theoretical framework set up back then and aims to further acquire new information about the PAQR-2 signaling pathway through systematic genetics, lipidomics and membrane property assays. Importantly, an additional aim is now to begin translating the knowledge obtained from *C. elegans* to mammalian cells in order to understand the role of the AdipoRs in fatty acid desaturation and membrane fluidity regulation. The model below provides a summary of what was known regarding the PAQR-2 pathway in *C. elegans* before I started my PhD in 2015 (Fig. 7).
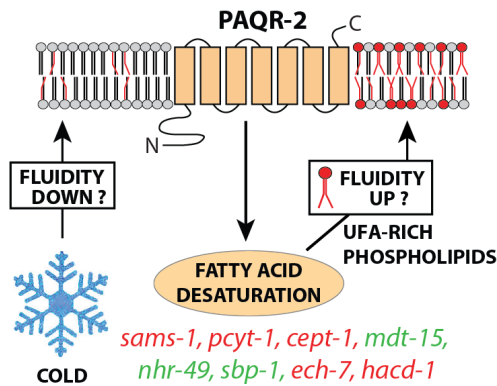
Fig. 7. PAQR-2 regulates fatty acid desaturation to maintain membrane fluidity at low temperatures. A proposed model suggesting that PAQR-2 regulates fatty acid desaturation as an adaptation response to prevent cold-induced membrane rigidification. Mutants lacking PAQR-2 fail to induce the desaturation response upon cold treatment; this can be rescued by mutations that promote fatty acid desaturation (in green: gain of function mutants; in red: loss of function mutants) to generate unsaturated fatty acid (UFA)-rich phospholipids and thus maintain membrane fluidity (adapted from (164)).

METHODS

The following sections contains a brief description of key methods that I used when contributing for the publications listed earlier.

C. elegans growth and maintenance

The wild-type N2 and all other mutant strains were grown and maintained at 20°C on NGM plates seeded with *E. coli* OP50 bacteria. OP50 was maintained on LB plates and re-streaked every 3-4 months from glycerol stocks frozen at -80°C. Single colonies were picked and cultured overnight at 37°C in LB medium and then used to seed NGM plates. Prior to all experiments, the worms were bleach-synchronized and their eggs allowed to hatch overnight in M9 buffer at 20°C. L1 larvae were then spotted into plates the following day.

Preparation of glucose plates

Plates containing a desired concentration of glucose (20 mM in all the experiments unless otherwise mentioned) plates were prepared by adding glucose (from filter-sterilized 1M stock solution) to cooled autoclaved NGM. These plates were allowed to solidify and then seeded with fresh OP50 cultures the following day.

Preparation of fatty acid plates

All the experiments related to fatty acid supplementation was performed in peptone-free NGM plates. Stock solution of palmitic acid (0.1 M) and oleic acid (0.5 M) were prepared in ethanol, further diluted in LB media, inoculated with OP50 bacteria and then cultured overnight at 37°C shaker. The following day, the bacterial culture was centrifuged, concentrated, washed twice and re-suspended in M9 buffer. The resulting bacterial solution was then seeded onto NGM plates lacking peptone (200 µl/plate), allowed to dry and worms were added the following day.

EMS-Mutagenesis

The worms (N2 or mutant strains, depending upon screening strategy) were grown until most reached the L4-stage. They were washed, collected, resuspended in M9 buffer and mutagenized with 0.05 M ethyl methane sulfonate (EMS) solution for 4 hours as per published protocols (165). After mutagenesis, the worms were washed twice with M9 buffer and transferred onto a new NGM plate and allowed to recover. Healthy looking late L4 hermaphrodites were individually picked and used as the parental generation (P0s).

Growth and tail-tip scoring assays

Length measurement was used to quantify the growth of the worms in all our experiments. For length measurement assay, synchronized L1s were spotted into normal NGM or other test plates and were allowed to grow for 72 hours or 144 hours (only for experiments related to 15°C). The worms were then individually picked, mounted onto microscopic slides, photographed and the length of at least 20 worms were manually measured using ImageJ. Tail-

tip scoring assay was performed on synchronous 1-day adults, i.e. 72 hours post L1, and the frequency of worms with wild-type or mutant tails was quantified.

Quantification of *pfat-7::gfp* expression

The HA1842 strain carrying *pfat-7::gfp* was a gift from Amy K. Walker (64). The reporter was crossed with the mutant strains and the gfp intensity was quantified on synchronized L4 worms using ImageJ.

Plasmids

The plasmids used for the experiments were generated using a Gibson assembly cloning kit (NEB). Different fragments were initially PCR amplified, purified and then ligated following the instructions from the company. The plasmids were then microinjected into worms at 10-25 ng/µl concentration together with an injection marker.

Lipidomics: Sample Preparation

Bleach-synchronized worms were initially grown on normal NGM plates for 30 hours and then further cultivated overnight under different treatments and finally harvested as L4 larvae (one 9 cm plate/sample). The worms were washed three times with M9 buffer, pelleted and finally stored at -80°C until further processing.

FRAP

A membrane-bound prenylated GFP reporter (*pGLO-1::GFP-CAAX*) expressed in intestinal cells (166) was used to perform all the FRAP experiments. The experiments were carried out using a Zeiss LSM700inv laser scanning confocal microscope with a 40X water immersion objective. Images were acquired at a 12-bit resolution over 256×256 pixels with a pixel dwell time of 1.58 µsec. Ten pre-bleaching images of gfp-positive membranes were acquired and then a certain region of interest (seven-pixel radius) was bleached using 488 nm laser with 50% of laser power. The fluorescence recovery was traced for 48 sec. A detailed protocol for performing FRAP experiments in *C. elegans* (167) is attached as an appendix to this thesis, since this is a recurring method used for the measurement of membrane fluidity.

FRET

For FRET experiments, strains carrying *pPAQR-2::GFP* (used as donor strain), *pIGLR-2::mCherry* (used as an acceptor strain) and *pPAQR-2::GFP; pIGLR-2::mCherry* (used as experimental strain with both donor and acceptor fluorophore) were used. The worms were bleach-synchronized and after 48 hours, they were washed and transferred onto non-peptone plates seeded with OP50 or OP50 pre-loaded with different fatty acids. After 24 hours, day 1 adult worms were individually picked onto a microscopic glass slide and immobilized using 10 mM levamisole. For experiments related to temperature, worms already mounted on the slides were incubated in the live cell chamber of the microscope for 30-45 mins. FRET images were acquired with an LSM880 confocal microscope equipped with a live cell chamber and ZEN software (Zeiss) using a 40X water immersion objective. To acquire GFP images, the worms were excited using 488 nm laser with 2% laser intensity and the emission was recorded between

494 and 565 nm. For images in mCherry channel, 561 nm laser with 0.2% laser intensity was used for excitation and the emission was recorded between 588 and 696 nm. Finally, to obtain FRET images, 488 nm laser (gfp-specific) with 2% laser intensity was used for excitation and the emission was recorded between 588 and 696 nm (mCherry-specific). All the images were acquired with 16 bits image depth and 1068x1068 resolution using a pixel dwell of 1.97 µsec. Images were further analyzed PixFRET plugin (168) in ImageJ.

RESULTS AND DISCUSSION

This thesis reflects a progressive study of the adiponectin receptor homolog PAQR-2 in *C. elegans*. Pathway elucidation using forward genetics has been a strength of the Pilon lab and in Paper I, we followed-up from 2013 (38) with another screen, but this time, for mutants that genocopy (169) *paqr-2.* In Paper II, we finally translated our findings from *C. elegans* into a mammalian system and showed that PAQR-2 (in *C. elegans*) and the AdipoRs (in mammalian cells) are essential to prevent dietary SFA-induced membrane rigidification. We followed that up with another study in Paper III showing that membrane fluidity regulation by the AdipoRs is cell non-autonomous. In Paper IV and V, we returned to forward genetics and screened for mutants that suppress the toxic effects of dietary SFAs observed in the *paqr-2* mutant and other different genetic backgrounds. Finally, in Paper VI, we asked the question 'What molecular mechanisms are specifically required to tolerate dietary saturated fats?' and performed yet another forward genetics to isolate mutants that fail to tolerate SFAs i.e. mutants that are either lethal or sterile when supplemented with a SFA-rich diet. To summarize, by leveraging a fundamentally unbiased experimental approach, we have been able to conclude that the primary cellular function of the AdipoRs is to guard membrane homeostasis, mostly by adaptively promoting fatty acid desaturation to maintain membrane fluidity. All the papers will be discussed in detail in the sections below.

PAPER I: *Caenorhabditis elegans* PAQR-2 and IGLR-2 Protect against Glucose Toxicity by Modulating Membrane Lipid Composition

RESULTS

iglr-2 is a *paqr-2* genocopier

We began this study with an unbiased forward genetic screen (Fig.8) to isolate mutants that genocopy (169) *paqr-2,* i.e. mutants that have phenotypes identical to those of the *paqr-2* mutant. Such a screen could possibly lead to the isolation of genes that act in the same pathway as PAQR-2, such as interaction partners, activators, co-factors or any eventual ligand. Wild-type N2 worms were mutagenized using EMS (ethyl methane sulfonate) and further grown at 20°C. The F2 generation were screened in two different ways: 1) Screened initially for mutants displaying the tail-tip phenotype characteristic of the *paqr-2* mutant and then further screened for the inability to grow at 15°C; and 2) Screen initially for mutants unable to grow at 15°C and, after recovering them at 20°C, scoring for the tail-tip phenotype. Combining both approaches, a total of over 80 000 haploid genomes were screened that resulted in the isolation of five *paqr-2* genocopier mutants: three turned out to be novel alleles of the gene *iglr-2*, and two were novel alleles of *paqr-2* itself. *iglr-2* alleles are likely *lof* mutations since two out of three (*et34* and *et38*) result in premature stop codons while the *et37* allele substitutes a neutral glycine with an aspartate in the single transmembrane domain.

IGLR-2 is structurally similar to LRRIG (leucine rich repeat and Ig containing proteins) proteins and is predicted to consists of a single transmembrane domain, an intracellular C-terminal domain and an extracellular immunoglobulin (Ig)-like domain followed terminally by five leucine rich repeats (LRRs) (170). There are nearly 40 LRRIG-type proteins in mammalian genomes, and they are known to have a variety of functions. Structurally, IGLR-2 is most similar to the mammalian AMIGO or LINGO proteins (both have a single Ig domain, similar numbers of LRRs and a short cytoplasmic domain). AMIGO acts as an anchor protein that facilitates the clustering and activity of neuronal potassium channel Kv2.1 (171, 172), while LINGO1 is part of a signaling complex involved in axon regeneration (173) and a known negative regulator of receptor tyrosine kinases, a function shared by other LRRIG proteins such as LRIG1 (174–177).

paqr-2 and *iglr-2* mutants are glucose intolerant and are indistinguishable from each other

During the mutant characterization, we noticed that both the *paqr-2* and *iglr-2* mutants were sensitive to sugars; the mutants were especially sensitive to the inclusion of glucose in the culture plates. Supplementing the culture plates with as little as 16 mM or 20 mM glucose had no visible effect on N2 worms but both *paqr-2* and *iglr-2* mutants failed to grow, arrested and eventually died as L2 larvae. This was particularly interesting since the mammalian AdipoRs have been shown to have anti-diabetic functions and important for glucose tolerance (132, 159). It is important to note that the glucose sensitivity phenotype observed in the *paqr-2* and *iglr-2* mutants is specific to D-glucose. L-glucose, a biological inert enantiomer of the natural D-glucose, did not have any effect which suggests that the mutants have a biological and metabolic response to glucose rather than being sensitive to some purely chemical or biophysical effect. The effects of glucose on *paqr-2* and *iglr-2* mutants was reversible within the initial 3 hours of exposure but not after longer treatments.

The *paqr-2* and *iglr-2* mutants have been indistinguishable in all of our assays. Along with having a similar glucose-intolerant phenotype, they both have a characteristic tail-tip morphology defect, similar cold-sensitivity, excess of SFAs in their membrane phospholipid, decreased expression of a *pfat-7::GFP* reporter as well as reduced brood size and growth rate. The double mutant *paqr-2 iglr-2* exhibits the same phenotypes and severity as either single mutant, which strongly suggests that the PAQR-2 and IGLR-2 proteins act in the same pathway. The fact that they are both transmembrane proteins also suggests that they might work together as a complex.

IGLR-2 interacts with PAQR-2 on plasma membranes

An *IGLR-2::GFP* translational reporter showed that IGLR-2 is predominantly and reproducibly expressed on the membranes of the gonad sheath cells (weak expression is also detected in head and tail neurons); an expression profile similar to that of *PAQR-2::GFP*. This suggests that PAQR-2 and IGLR-2 may act together in gonad sheath cells. We also noticed that PAQR-2 localization to the gonad sheath cells was dependent on IGLR-2 as there was a reduction in the expression of the *PAQR-2::GFP* reporter on the gonad sheath cell membranes

in *iglr-2* mutants. We further used BiFC to test the hypothesis that PAQR-2 and IGLR-2 actually interact in the cell membranes. BiFC is a method commonly used to visualize protein interactions in living cells, and is based on the reconstitution of complementary fragments of a fluorescent protein fused to two potential interacting partners. The interaction between the two candidate proteins allows the close proximity, assembly and fluorescence of the fragments of the fluorescent protein (178, 179). Using BiFC, we showed that PAQR-2 and IGLR-2 indeed interact in the intestinal membrane, where expression was driven by an intestine-specific heat shock promoter (180, 181).
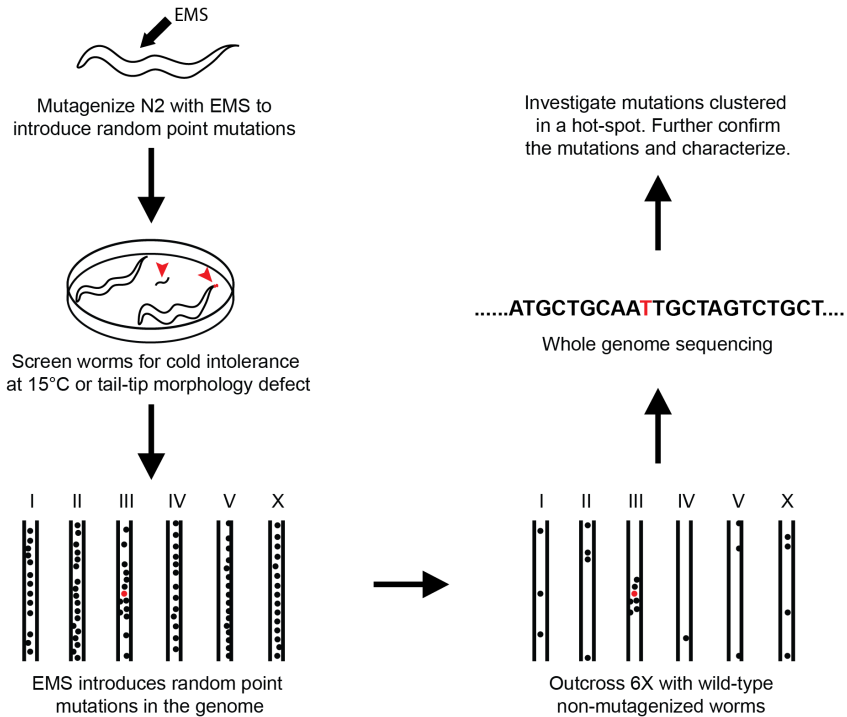
Fig. 8. Schematics of the forward genetics screen to isolate and molecularly define *paqr-2* genocopiers. Wild-type N2 worms were mutagenized with EMS, grown at 20°C and the F2 generation was screened for the tail-tip morphology defect and cold intolerance at 15°C. Worms that exhibited both these phenotypes were identified, outcrossed 6 times to wild-type non-mutagenized worms to clean the genome (since EMS introduces random mutations), then had their genome sequenced. Mutations within clusters of homozygosity were investigated and further characterized to identify actual causative mutations.

Mutations that promote fatty acid desaturation can rescue the glucose sensitivity

In order to understand the mechanisms of glucose toxicity in *paqr-2* and *iglr-2* mutants, we further tested whether previously identified genetic suppressors of the cold sensitivity and

tail tip defects of *paqr-2* mutants (38) could also suppress the glucose intolerance phenotype. The *lof* mutants in the β-oxidation pathway (*ech-7* and *hacd-1*) had no effect on the glucose sensitivity phenotype of the *paqr-2* mutant. An independent study had shown that mutations in *acdh-11,* an acyl-CoA dehydrogenase that acts upstream of *ech-7* and *hacd-1* in the β-oxidation pathway, could also act as a *paqr-2* suppressor (40). The *acdh-11 lof* mutant indeed could rescue the cold sensitivity and tail tip phenotypes but did not rescue the glucose intolerance of *paqr-2*, and thus behaved precisely as the *ech-7* and *hacd-1* mutations. Mutations in genes involved in PC synthesis pathway (*pcyt-1(et9)*, *cept-1(et10* and *11)*) partially suppressed the glucose intolerance with ~20% of the worms developing into fertile adults. Finally, the best possible suppression was from the *gof* mutants *nhr-49(et8)* and *mdt-15(et14)* as well as SBP-1 overexpressing transgene, where ~80% of the worms developed into fertile adults even when cultivated in the presence of glucose. These suppressors could also suppress the glucose intolerance phenotypes of *iglr-2* and *paqr-2 iglr-2* double mutant. These genetic interaction studies suggest that PAQR-2 and IGLR-2-dependent regulation of Δ9 desaturases are important for worms to tolerate glucose since mutations that promote fatty acid desaturation can partially bypass the need for PAQR-2 and IGLR-2 to survive on glucose.

Glucose increases membrane rigidity in *paqr-2* and *iglr-2* mutants

We then tested whether glucose supplementation affects membrane fluidity in *paqr-2* and *iglr-2* mutants. Using the method of Fluorescence recovery after photobleaching (FRAP), we measured membrane fluidity directly *in vivo* at 20°C, 15°C and also on glucose-treated condition. In the FRAP experiment, a high-powered laser is used to bleach fluorophores on a small region of the membrane, and the recovery of fluorescence into this bleached region from the non-bleached surrounding regions is recorded and quantified (182). We performed FRAP experiments on the worms expressing prenylated, membrane-bound GFP in the intestine (166) and found that, at 15°C and under glucose-treated conditions, *paqr-2* and *iglr-2* mutants had reduced membrane fluidity; wild-type worms displayed the same membrane fluidity under all conditions. We did not detect any measurable difference in membrane fluidity among wild-type, *paqr-2* and *iglr-2* mutants cultivated in control condition. As with other phenotypes, the reduced membrane fluidity in *iglr-2* mutant treated with glucose was efficiently suppressed by the *gof* allele *nhr-49(et8),* which further suggests that upregulation of desaturase expression to maintain fluidity is a mechanism required to counter potential glucose toxicity. Increased membrane rigidity in the *paqr-2* and *iglr-2* mutants under glucose-treated condition was consistent with a strong increase in the levels of SFAs in PCs and PEs. A further evidence supporting the important role of membrane fluidity homeostasis during glucose challenges is the observation that including small amount of non-ionic detergents in the culture plates, which likely increases membrane fluidity (183), could partially suppress both the growth and membrane fluidity defects observed in *paqr-2* and *iglr-2* mutants.

IGLR-2 acts cell nonautonomously

As already mentioned, PAQR-2 and IGLR-2 are predominantly and reproducibly expressed in the membranes of the gonad sheath cells. Our FRAP experiments performed in the intestinal cells suggests that worms lacking PAQR-2 and IGLR-2 cannot maintain membrane fluidity in the intestine in cold or in the presence of glucose. Further, our whole worm lipidomics analysis have shown differences in the phospholipid composition both on normal condition and when cultivated in the presence of glucose. These results suggest that PAQR-2 and IGLR-2 regulate membrane homeostasis cell nonautonomously. To further study the site of function of *iglr-2*, we performed a mosaic analysis using the *SUR-5::GFP(NLS)* reporter, which is ubiquitously expressed in the nuclei of all the cells and had been previously used for mosaic analysis (184). Mosaic analysis is based on the principle that the transgenes retained as extrachromosomal arrays are not always segregated to both daughter cells upon cell division and hence are occasionally lost from some cells during embryogenesis, resulting in genetic mosaics (185). This is an unbiased approach to study and understand the tissue specificity of any particular transgene. We scored *iglr-2* mutants carrying an extrachromosomal array harboring an *iglr-2*-rescuing transgene along with *SUR-5::GFP(NLS)*: only worms carrying the array in tissues able support systemic *iglr-2* function would be able to grow to become fertile adults on 20 mM glucose. We found that the presence of the *iglr-2* rescuing transgene only in the ABa or ABp lineage, specifically in cells of the hypodermis, was sufficient to rescue the glucose intolerance phenotype of the *iglr-2* mutants. This suggests that the hypodermis is a sufficient site of IGLR-2 function but does not exclude the possibility that IGLR-2 may also have an important function in the gonad sheath cells where its GFP tagged version is visibly localized. Mosaic analysis on PAQR-2 and further cell nonautonomous role of PAQR-2 and IGLR-2 will be described in more details in Paper III.

DISCUSSION

In this study, we have identified a new interacting partner for PAQR-2. Our results indicate that PAQR-2 and IGLR-2 interact and function together to regulate fatty acid desaturation, possibly in the hypodermis and/or gonad sheath cells. We also show that supplementing glucose in the culture plates lead to the accumulation of excess SFAs in the membrane phospholipids, resulting in reduced membrane fluidity unless PAQR-2 and IGLR-2 are functional. Further, glucose-induced membrane rigidification can be suppressed by mutations that promote fatty acid desaturation or by the inclusion of non-ionic detergents at membrane-fluidizing concentrations in the culture plates.

The effect of glucose in *C. elegans* has been mostly studied in the context of longevity and aging (122, 124–126). Supplementing glucose in the culture plates affects the lipid composition in *C. elegans*. In particular, glucose increases the levels of total TAGs (122, 126, 129) and also SFAs even though worms respond by upregulating the expression of Δ9 desaturases (186). This suggest that supplementing glucose activates *de novo* lipogenesis, a lipid synthesis pathway that relies on acetyl-CoA as a substrate to produce SFAs and other lipids. It is also possible that glucose leads to changes in dietary fatty acid composition since

the dietary *E. coli* OP50 can utilize glucose and convert it to fatty acids (187). Increase in glucose-induced SFAs in wild-type worms do not affect their growth and membrane fluidity, reinforcing the importance of PAQR-2 and IGLR-2 in maintaining membrane composition and fluidity within the physiologically acceptable range. It has also been shown that the glucose-mediated increase in SFAs and subsequent decrease of MUFAs and PUFAs is exacerbated by RNAi against *sbp-1* and *mdt-15*. This is consistent with the fact that inhibition of *sbp-1* and *mdt-15* lead to downregulation of the *fat-7* Δ9 desaturase (126). We have now observed a similar phenomenon in the *paqr-2* and *iglr-2* mutants, which again suggests that PAQR-2/IGLR-2 likely regulate fatty acid desaturation by activating downstream effectors such as *sbp-1, mdt-15* or *nhr-49*.

It is interesting that the glucose toxicity observed in the *paqr-2* and *iglr-2* mutants can be suppressed by providing a small amount of non-ionic detergents in the culture plates. Not only membrane fluidity but also the growth in presence of glucose is partially rescued by mild detergents. This again, and independently of the genetic studies, suggests that glucose-induced membrane rigidification is a phenomenon deserving special attention, especially in the context of diabetes. Chronic hyperglycemia during the progression of type II diabetes have been shown to cause oxidative stress, apoptosis of pancreatic β-cells (188), lipotoxicity (189) and ultimately dysfunction and failure of different organs (190). These are some of the proposed glucose toxicity mechanisms, though none is universally accepted. Chronic hyperglycemia has also been associated with increased membrane rigidity in erythrocytes (191, 192). Therefore, the cascade of metabolism from glucose to lipids and subsequent influence on membrane properties can be an avenue for further study to better understand the toxic effect of glucose in diabetes.

To conclude, we speculate a model (Fig. 9) in which cold- or glucose-induced membrane rigidification activate PAQR-2 and IGLR-2 that together act as a fluidity sensor, thereby promoting fatty acid desaturation via activating downstream effectors such as NHR-49, MDT-15 or SBP-1 to restore membrane composition and fluidity. Many important questions remained at this time, including: 1) What is the mechanism of fluidity sensing?; 2) How does PAQR-2/IGLR-2 activate the downstream effectors, i.e what is the nature of the signal?; and 3) Is PAQR-2-mediated membrane fluidity regulation conserved in mammalian cells?
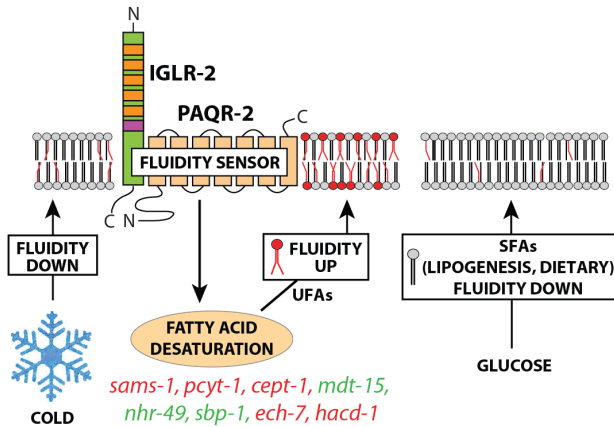
Fig. 9. PAQR-2 and IGLR-2 regulates membrane fluidity. A proposed model suggesting that when membranes are challenged with conditions that lead to increased rigidity, either by lower temperature, or by the presence of glucose, which leads to increased SFAs (either dietary or via lipogenesis), the PAQR-2/IGLR-2 complex becomes activated (i.e. acts as a fluidity sensor) and prevents membrane rigidification by promoting fatty acid desaturation. Mutants that lack PAQR-2 and IGLR-2 hence fail to induce the desaturation response when cultivated in cold or in the presence of glucose; this can be rescued by secondary mutations (*gof* alleles in green; *lof* alleles in red) that promote fatty acid desaturation to generate increase the proportions of UFAs and that ultimately restores membrane fluidity.

Own Contributions to Paper I

1. Mosaic analysis experiments: Co-injected the *SUR-5::GFP(NLS)* reporter with *iglr-2* rescuing transgene and performed mosaic analysis experiments.
2. Subset of FRAP experiments (Treatment with detergent NP-40): Only this particular set of experiment was done by me.
3. Growth experiments in glucose: I performed and analyzed the growth of novel alleles of *paqr-2* and *iglr-2* in the presence of glucose.
4. Microscopy (Images of *PAQR-2::GFP* and *IGLR-2::GFP*): Performed crosses to generate *paqr-2* mutant carrying *IGLR-2::GFP* transgene and *iglr-2* mutant carrying *PAQR-2::GFP* transgene, acquired the images and scored the frequency of worms expressing gfp in the gonad sheath cells.
5. Microinjection (Test the effect of *rol-6* injection marker): Injected *rol-6* marker and scored whether the "roller" phenotype affects the length of the worms.

PAPER II: The Adiponectin Receptor AdipoR2 and its *Caenorhabditis elegans* homolog PAQR-2 Prevent Membrane Rigidification by Exogenous Saturated Fatty Acids

RESULTS

Metabolites in glycolysis pathway are toxic to *paqr-2* mutant

This study was initiated with the aim of understanding the mechanism of glucose toxicity in the *paqr-2* mutants. Glucose supplementation leads to an increase in the level of SFAs, which becomes aggravated in *paqr-2* mutant, causing membrane rigidity (164). As mentioned earlier, the metabolism of glucose to SFAs probably occurs via the glycolysis pathway leading to *de novo* lipogenesis. Here, we tested this hypothesis and found that not only glucose but supplementation of other metabolites within the glycolysis pathway, such as glycerol, dihydroxyacetone, pyruvate and lactate, were also toxic to *paqr-2* mutant. Incidentally, it is interesting to again point out that both glycerol and dihydroxyacetone have been previously associated with glucose toxicity in *C. elegans* (124, 126). In our own study, we noticed that, similar to glucose, glycerol and pyruvate also increased membrane rigidity in the *paqr-2* mutant. These results suggest that glucose metabolism via the glycolysis pathway plays a major role in glucose-mediated toxicity in the *paqr-2* mutant.

Dietary *E. coli* mediates the toxicity of metabolites

Even though glucose is thought to have an effect directly within the worm to affect its life-span and longevity (122, 124), the possible involvement of the dietary *E.coli* OP50 in glucose uptake and metabolism should also be considered, as others also previously noted (129). We wanted to specifically test this since the glucose concentration used in our study (20 mM or 0,4%) is much lower rather than concentrations used in life-span studies (111 mM or 2%). In a first series of critical experiments, we found that the *paqr-2* mutant grown on ΔPTS *E. coli,* a mutant *E. coli* strain incapable of glucose uptake (124), was no longer sensitive to glucose supplements. This clearly demonstrates that the effect of glucose is not directly on the *paqr-2* mutant worms but is instead mediated by the dietary *E. coli* and requires uptake by these bacteria. To further understand the metabolic fate of the glucose that is taken up by the bacteria, we used deletion mutants from the Keio collection (193), specifically focusing on mutations in genes that are involved in glycolysis and fatty acid synthesis. When fed the parental BW25113 *E. coli* strain, from which the Keio library is generated, the *paqr-2* mutant continued to display its characteristic cold sensitivity and tail tip defects. However, only glucose and glycerol but not dihydroxyacetone, pyruvate and lactate were toxic to the *paqr-2* mutant when cultivated on the BW25113 diet. Further, mutants from the Keio collection in genes involved in glucose and glycerol metabolism attenuated or completely abolished the toxic effects of glucose and glycerol on the *paqr-2* mutant. This suggests that an output from the bacterial glucose metabolism has a profound impact on the *paqr-2* mutant worm phenotype. Note that wild-type worms grew well in all culture conditions, or when using the Keio mutants as the food source.

We hypothesized that *E. coli* mutants unable to metabolise glucose would be unable to convert it into SFAs, which led to our next series of experiments.

Diets with high SFA/MUFA ratios are toxic to the *paqr-2* mutant

In order to understand whether *de novo* lipogenesis actually occur in the bacteria, we performed lipidomics and analyzed the fatty acid composition in different bacterial strains cultivated in the presence of glucose, glycerol and pyruvate. We found that the ratio of even-chain SFA-to-MUFA, or specifically the ratio of 16:0 to 18:1n-7, was dramatically increased when OP50 *E. coli* was cultivated in the presence of glucose, glycerol or pyruvate. The ΔPTS OP50, a mutant unable to uptake glucose, had a higher even-chain SFA to MUFA ratios when cultivated in the presence of glycerol and pyruvate but not in the presence of glucose. And finally, BW25113, the Keio parental strain, had a higher even-chain SFA-to-MUFA ratio when cultivated in the presence of glucose or glycerol but not when cultivated in the presence of pyruvate. These results perfectly correlated with our observation that the toxicity of these metabolites in the *paqr-2* mutant is completely dependent on the bacterial diet: any bacteria/culture condition that led to elevated SFA/MUFA ratios within the dietary bacteria is toxic to the *paqr-2* mutant. Further, diets with increased SFA/MUFA ratios also led to the accumulation of excess SFAs in the worm phospholipids, a phenomenon strongly exacerbated in *paqr-2* mutants where the excess SFAs in phospholipids correlate with membrane rigidification and lethality.

PAQR-2 is essential to tolerate exogenous SFAs

Our observation regarding glucose-mediated increase in dietary SFAs being toxic to the *paqr-2* mutant led us to test the effect of SFAs directly. We developed and optimized a protocol to pre-load *E. coli* with PA and noticed that bacteria incorporated PA very efficiently into their membrane phospholipids. PA-loaded *E. coli* was extremely toxic to the *paqr-2* mutant; wild-type worms were unaffected. Cultivating worms in PA-loaded *E. coli* led to a dramatic accumulation of PA in both PCs and PEs in worms, a phenotype that was aggravated and led to increased membrane rigidity in the *paqr-2* mutant. Consistently, we found that wild-type worms responded by PAQR-2-dependent upregulation of FAT-7 desaturase expression that likely countered the toxic effects of SFAs by generating MUFAs and PUFAs. This was further supported by an experiment where pre-loading the bacterial diet with a combination of both PA and OA reduced the toxic effect of PA on growth and membrane rigidification in *paqr-2* mutant. Altogether, these results suggest that PAQR-2 regulates fatty acid desaturation, maintains the SFA/MUFA ratio and prevents cell membrane rigidification induced by dietary SFAs.

AdipoR2 prevents membrane rigidification induced by exogenous SFAs

Culturing mammalian cells in the presence of PA increases membrane rigidification (194). We now tested whether our findings concerning PAQR-2-mediated regulation of membrane homeostasis were conserved in mammalian cells. To this end, we cultured human

embryonic kidney (HEK293) cells and found that addition of 400 μM PA indeed led to an increase in membrane rigidification, a cellular phenotype that could be suppressed by the inclusion of OA. To specifically test the possible role of the AdipoRs in preventing membrane rigidification by exogenous SFAs, we optimized efficient knockdown of AdipoR1 and AdipoR2 using siRNAs and found that inhibition of AdipoR2, but not of AdipoR1, exacerbated membrane rigidification in HEK293 cells treated with PA, even when provided at such low doses as 100 μM or 200 μM, which have no effect on control cells. Knockdown of AdipoR1 or AdipoR2 had no effect on the membrane fluidity under normal culture conditions (i.e. without PA supplement). We further analyzed the lipid composition in HEK293 cells and found that knockdown of AdipoR1 and AdipoR2 resulted in an accumulation of SFAs in PCs, PEs and TAGs in control conditions. Additionally, the effect of PA on the membrane SFA content was much more pronounced when AdipoR2 was knocked down. Other previously suggested that AdipoR2 may act as a ceramidase (146, 147), and we therefore also analyzed ceramide levels in our cells. We found that treating cells with PA increased the relative levels of ceramides (not surprising since the PA supply increases ceramide synthesis), but that AdipoR2 knockdown actually caused a reduction in total ceramide levels, which was somewhat surprising if AdipoR2 is a ceramidase. Our main conclusion from these results is that mammalian AdipoR2, like the worm PAQR-2, is essential to prevent membrane rigidification following exposure to exogenous SFAs (Fig. 10).

DISCUSSION

Given the enormous variation in the fatty acid content/composition of different diets, and the fact that the dietary fatty acids are readily incorporated into cellular membranes, a regulatory mechanism to adjust the membrane composition and properties must be of critical importance. *De novo* lipogenesis is coordinated with desaturation (195), most likely in order to facilitate membrane homeostasis, but a precise regulatory pathway that responds to dietary fats has not yet been described. This study highlights one such regulatory mechanism where PAQR-2 in *C. elegans* and AdipoR2 in mammalian cells protect against dietary SFA-mediated membrane rigidification. In *C. elegans,* 70% of the membrane phospholipids are renewed within a day in a post-reproductive adult (55) and almost 80% of the newly incorporated fatty acid are either directly obtained from the diet or modified (e.g. elongated/desaturated) versions of dietary FAs (80). Further, the role of Δ9 desaturases (*fat-5*, *fat-6* and *fat-7*) in *C. elegans* is critical to determine membrane composition and turnover (55, 196). Through our work we have now identified PAQR-2 as a regulator of desaturases in response to dietary fatty acids that is critical for the maintenance of membrane homeostasis. At this stage of our work still did not know how PAQR-2 could sense defects in membrane composition/properties but we did speculate that membrane composition could influence the interaction between PAQR-2 and its partner IGLR-2 (164) and in this way regulate the activation of downstream desaturation processes.

Here, we have finally shown that membrane homeostasis is an evolutionary conserved function of the PAQR-2/AdipoR2 type of proteins. Our results with mammalian cells suggests that AdipoR2 regulates membrane fluidity independently of adiponectin, a proposed adipocyte-

secreted ligand (197, 198), since in our experiments we have used serum-free media (for basal conditions) or serum-free media supplemented with PA/PA+OA. Further, HEK293 cells are highly unlikely to secrete adiponectin since it is a protein exclusively secreted by adipocytes. We also note that there is no obvious homolog of adiponectin in *C. elegans* and that we did not find any candidate ligand in our genocopy screen (164). We therefore conclude with some confidence that a ligand is not required for AdipoR2-dependent regulation of membrane composition and fluidity in HEK293 cells. However, it remains possible that supplementation of adiponectin could further enhance the effect of AdipoR2, a phenomenon observed by others (146, 147).
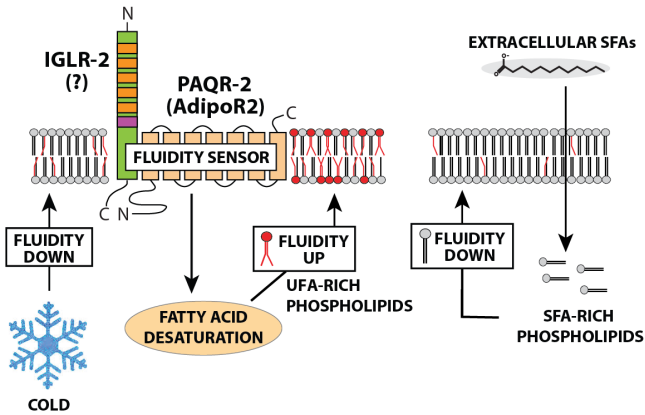

Fig. 10. PAQR-2/AdipoR2-mediated regulation of membrane fluidity in the presence of exogenous SFAs. A proposed revised model that shows how membrane rigidification induced by exogenous SFAs (in both worms and cells) or cold (in worms) activates the PAQR-2/IGLR-2 complex in *C. elegans*, or AdipoR2 in human cells, resulting in the activation of downstream desaturases to generate UFA-rich phospholipids that promotes membrane fluidity.

Our findings could help understand the roles of the AdipoRs in mammalian physiology. In particular, others have shown that AdipoR1 and AdipoR2 knockout mice exhibit defects in lipid homeostasis (153, 154, 156, 157, 199). Conversely, inducible overexpression of the AdipoRs enhances glucose metabolism and hepatic insulin sensitivity and, on a leptin-deficient genetic background, overexpression of AdipoR2 suppresses the diabetic phenotypes (200). In human, a mutation in AdipoR1 has been associated with autosomal dominant retinitis pigmentosa (201), a phenotype attributed to AdipoR1-dependent uptake, retention and elongation of DHA in photoreceptors and that is adiponectin independent (199). Further, in diabetics, there has been reports of increased SFA levels and membrane rigidity (191, 202–204), which could be a result of conversion of glucose to SFAs via *de novo* lipogenesis (205); our new findings suggest the possibility that chronic elevated conversion of glucose to SFAs in diabetics could overwhelm AdipoR-dependent membrane homeostasis. In the future, it would be interesting to thoroughly study the relationship between hyperglycemia and dyslipidemia,

and to specifically determine whether AdipoR2-mediated regulation of membrane fluidity can explain, or actually prevent, some aspects of the pathologies seen in diabetic patients

Own Contributions to Paper II

1. Growth experiments: Performed and scored the growth of the wild-type and *paqr-2* mutant in the presence of different glycolysis-related metabolites or when treated with other stressors. Also established and optimized the protocol to pre-load bacteria with PA and performed experiments to test the effect of dietary SFAs on the growth of wild-type and *paqr-2* mutant.
2. FRAP experiments: Performed and analyzed whether different metabolites, stressors and dietary fatty acids affect membrane fluidity.
3. *C. elegans* lipidomics sample preparation: Prepared worm samples for lipidomics (We harvest worms in L4 stage and then send it to our collaborators for further processing)
4. *pfat-7::GFP* expression: Acquired images and quantified expression of *pfat-7::GFP* reporter.

PAPER III: Membrane Fluidity is Regulated Cell Nonautonomously by *Caenorhabditis elegans* PAQR-2 and its Mammalian Homolog AdipoR2

RESULTS

PAQR-2 regulates membrane fluidity cell nonautonomously

In paper I, we performed a small-scale mosaic analysis to understand the site of action of IGLR-2 and found that the expression of IGLR-2 in the hypodermal tissue was sufficient to allow *paqr-2* mutant worms to grow on glucose (164). Here, we followed up on that study and performed a more extensive mosaic analysis (analyzing 50 worms that had lost the transgene from the intestine) to identify tissues where expression of PAQR-2 and IGLR-2 could be sufficient to rescue the mutant phenotypes. As mentioned earlier, mosaic analysis relies on the fact that transgenes retained as extrachromosomal arrays in *C. elegans* are often lost during cell divisions, which leads to genetically mosaic animals. Therefore, with the help of an appropriate marker, for example the SUR-5::GFP(NLS) reporter that can be expressed in the nuclei of any cell, one can determine the effect of cellular inheritance in particular cells of the functional transgene on the mutant phenotype (185). Our results from this more comprehensive mosaic analysis study suggest that the expression of *paqr-2* and *iglr-2* in either gonad sheath cells or hypodermis is sufficient to rescue the glucose intolerance phenotypes of the mutants. However, due to the strong intestinal SUR-5::GFP(NLS) expression that obscures visualization in other tissues (184), we specifically analyzed only worms that did not have gfp expression in the intestine.

To further verify our results from the mosaic analysis, and also test effects of intestinal-specific expression, we generated transgenic worms with *paqr-2* expression driven by tissue-specific promoters and subsequently scored their phenotypes. We found that specific expression of PAQR-2 in either hypodermis or gonad sheath cells indeed was sufficient to rescue the growth defects on glucose, the cold intolerance, and the tail tip morphology defect, as well as the reduced brood size phenotype usually observed in the *paqr-2* mutant. We also noticed that *paqr-2* expression specifically in intestinal cells could also rescue all the *paqr-2* phenotypes. Additionally, we found that specific expression of *paqr-2* in the hypodermal cells could rescue the membrane fluidity defects observed in the intestinal cells of the *paqr-2* mutant. This is a convincing evidence that PAQR-2 can regulate membrane fluidity cell nonautonomously. We also noticed that expression of *paqr-2* even in body wall muscles could partially rescue the reduced brood size and the tail tip morphology phenotype. This suggests that even expression in muscle could provide some benefits to achieve membrane homeostasis. However, the glucose intolerance and cold intolerance phenotype was not improved by muscle-specific *paqr-2* expression. All together, these results suggest that PAQR-2 contributes cell nonautonomously to membrane homeostasis.

paqr-2 acts through *fat-6* in the hypodermis

We had previously shown that PAQR-2 regulates fatty acid desaturation to prevent membrane rigidification by exogenous SFAs. In particular, we had studied the expression of the Δ9 desaturase FAT-7 and found that PAQR-2-dependent induction of FAT-7 desaturase is important to counter the membrane rigidifying effects of SFA-rich diets (131, 164). Here we tested whether *paqr-2* expression in different tissues could induce the expression of the *pfat-7::gfp* reporter in intestinal cells. We found that only *paqr-2* expressed from its own native promoter could significantly enhance the expression levels of the *pfat-7::gfp* reporter. This clearly shows that the rescuing ability of tissue-specific transgenes is not strictly dependent on increasing the levels of *pfat-7::gfp;* induction of other Δ9 desaturases may also be important. There are three Δ9 desaturases in *C. elegans* (*fat-5, fat-6 and fat-7*), which have been shown to be functionally redundant (35, 36). All three desaturases play a major role in maintaining overall membrane composition (55) and further, the suppression of *paqr-2* mutant phenotypes by mutants that promote fatty acid desaturation is dependent on both *fat-6* and *fat-7* (38).

FAT-5 is expressed in the intestine and pharynx, FAT-7 is exclusively expressed in the intestine (36), and FAT-6 is expressed in both intestine and hypodermis, a pattern that we reported in this study and that is consistent with earlier studies (36). Interestingly, the *paqr-2;fat-6* double mutant is sterile but the *paqr-2;fat-7* double mutant is viable and fertile (37). Since the *pfat-7::gfp* reporter was not upregulated in the *paqr-2* mutants rescued with various tissue-specific *paqr-2* transgenes, we explored the possibility that the ability of *paqr-2* transgenes to rescue the mutant phenotypes is instead dependent on *fat-6*. We found that hypodermis-specific expression of *paqr-2* did not rescue the sterility phenotype of *paqr-2;fat-6* double mutant but could rescue the glucose intolerance of *paqr-2;fat-7* double mutant. The expression of *paqr-2* in gonad sheath cells also failed to rescue the sterility of *paqr-2;fat-6* double mutant but could rescue the growth defects of *paqr-2* and *paqr-2;fat-7* double mutant on normal condition and on glucose-treated condition. However, intestine-specific expression of *paqr-2* was able to rescue all the *paqr-2* phenotypes even in the presence of either *fat-6* or *fat-7* mutations. These results suggest that hypodermal- or gonad sheath cells-specific *paqr-2* is dependent on *fat-6* for systemic rescue of the *paqr-2* mutant phenotypes. Since all the three desaturases are expressed in the intestine, *paqr-2* expression in the intestine is not particularly dependent on one or the other, but rather likely activates more than one desaturase in the intestine. Further, in a separate experiment we found that co-expression of both *paqr-2* and *fat-6* specifically in hypodermis could efficiently rescue the sterility, glucose intolerance and tail-tip defect in the *paqr-2;fat-6* double mutant. This result suggests that *paqr-2* likely regulates *fat-6* in the hypodermis and that this is required for its ability to rescue the *paqr-2* mutant phenotypes systemically.

paqr-2 and *iglr-2* regulate vitellogenin transport and uptake

Our results so far indicate that *paqr-2* expression in any large membranous tissue can achieve systemic membrane homeostasis in the entire worm. This likely relies on effective transport of lipids among various tissues. In *C. elegans,* the best understood lipid transport

machinery is that of the vitellogenins, which are functionally somewhat equivalent to mammalian lipoproteins and are synthesized by the intestine, secreted into the pseudo-coelomic cavity, transported through gonad sheath cells and eventually endocytosed by maturing oocytes via the RME-2 receptor (93). We experimentally explored whether *paqr-2* and *iglr-2* may have a role in lipid transport via vitellogenins. Through microscopy observations, we noted severe defects in the shape and organization of the germline in *paqr-2* and *iglr-2* mutants, especially in the pachytene region of the gonad, and also noticed the presence of a large number of apoptotic bodies. Interestingly, the germline defects were not observed at the L4 stage, i.e. the stage prior to vitellogenin synthesis. Using a *vit-2::gfp* reporter to visualize vitellogenins, we noticed a dramatic accumulation of vitellogenin in the pseudocoelom of the mutants. This defect in lipid transport in the *paqr-2* mutants likely contributes to its low brood size and germline defect. Further, the vitellogenin uptake defect could be rescued cell nonautonomously by expression of *paqr-2* in hypodermis, intestine, muscle, and cell autonomously by expression in gonad sheath cells. These results suggest that the transport of vitellogenins to the germline is dependent on *paqr-2* and *iglr-2*.

AdipoR2 regulates membrane fluidity cell non-autonomously

We then examined whether PAQR-2-dependent cell nonautonomous regulation of membrane fluidity and homeostasis is conserved in mammalian cells. For this purpose, we used four-quadrant partitioned plates to culture HEK293 cells and inhibited AdipoR2 in some quadrants using siRNA, then removed the partition and assayed membrane fluidity in all the quadrants. Our results showed that upon PA challenge, cells expressing AdipoR2 are able to prevent PA-induced membrane rigidification in distant cells with which they have no direct contact and where AdipoR2 is silenced. We also noticed this same phenomenon with SCD-expressing cells being able to prevent PA-induced membrane rigidification in distant cells lacking SCD. Thus, as with PAQR-2 and its downstream targets FAT-6/FAT-7 in *C. elegans*, mammalian cells expressing AdipoR2 likely respond to PA-induced membrane rigidification to induce the expression of SCD, and generate UFAs that can be further shared with distant cells to maintain membrane homeostasis. In a somewhat more rigorous experimental setup, we also tested whether cells can actually exchange lipids without cell to cell contact. For this purpose, we used transwell plates and found that the donor cells pre-treated with ^{3}H-labelled PA were able to deliver the labeled PA to acceptor cells from which they were separated by a membrane barrier; the observed lipid transport is likely dependent on the presence of albumin or serum in the culture media. In conclusion, and consistent with the *C. elegans* finding, our results from the mammalian cells confirms that AdipoR2 regulates membrane homeostasis cell nonautonomously.

DISCUSSION

Based on the results up to and from Paper III results, we proposed a revised model (Fig. 11), where the PAQR-2/IGLR-2 complex in the hypodermal membranes (or other large membranous tissue) is activated by membrane rigidification, then induces the expression of Δ9

desaturase (FAT-6 in hypodermis), thereby promoting the synthesis of MUFAs and PUFAs, which will likely be transported to normalize and achieve membrane homeostasis in all other tissues. This is further based on our observation that *paqr-2* expression in hypodermis is dependent on *fat-6,* not *fat-7,* and that co-expression of both *paqr-2* and *fat-6* in the hypodermis is sufficient to rescue all the phenotypes observed in *paqr-2;fat-6* double mutant. Importantly, the hypodermis is a major fat storing tissue in *C. elegans* (87, 206). Lipids are most likely transported through the pseudocoelomic fluid in *C. elegans* and may involve vitellogenins or other identified lipoprotein-like proteins (207). An overt-reliance on dietary saturated fats (80), accompanied with a rapid membrane turnover with almost 80% of phospholipids being replaced in a day (55) implies that lipids are likely in constant flux among *C. elegans* tissues.

It is further possible that the PAQR-2/IGLR-2 complex signals to the intestine to promote fatty acid desaturation. An alternative hypothesis is that activation of the proposed hydrolase/ceramidase activity in PAQR-2 (146, 147) could generate a signaling molecule that diffuse to the intestine to activate fatty acid desaturation in that tissue. The intestine could then secrete UFAs for further distribution to all other tissues. Communication between the intestine and other tissues is well-documented. For example, the hypodermis can modulate lipid accumulation in the intestine through a *sma-3*-dependent signal (208), the gonad has been shown to communicate to the intestine to regulate the vitellogenin production (209), and the intestine regulates germline sex determination via specific fatty acids (210). The signaling hypothesis can also explain our observation that co-expression of *paqr-2* and *fat-6* in the gonad sheath cells cannot rescue the sterility of *paqr-2;fat-6* double mutant. An additional alternative is that a putative signal resulting from PAQR-2 activity in the gonad sheath cells might further depend on *fat-6*-expressing tissues such as hypodermis or intestine.

One of the ways in which lipids are transported between cells or cellular compartments is through membrane contact sites (211). However, lipoproteins and albumins can also mediate the transport of fatty acids and lipids (212), extracellular vesicles are yet another important mediator of lipid transport (213). In our experiments, the presence of albumin likely accounts for the transport of UFAs between distant cells. Indeed, we have specifically observed that the transport of ^{3}H-labelled PA is dependent on the presence of albumin or serum in the culture media.

To summarize, the most important conclusion from this study is that cells can achieve membrane homeostasis cell nonautonomously, both in *C. elegans* and in mammalian cells. This type of cell nonautonomous regulation where a cell shares its resources with other cells aligns with the concept of 'ordered heterogeneity', a property where tissues sought to achieve a larger regularity and homogeneity even though there is increasing heterogeneity among the individual cells themselves (214). It would be interesting in the future to test whether these findings are relevant to understand the robustness of whole organisms in response to diseased states since healthy cells would continue to share and provide essential resources to the impaired cells or tissues.
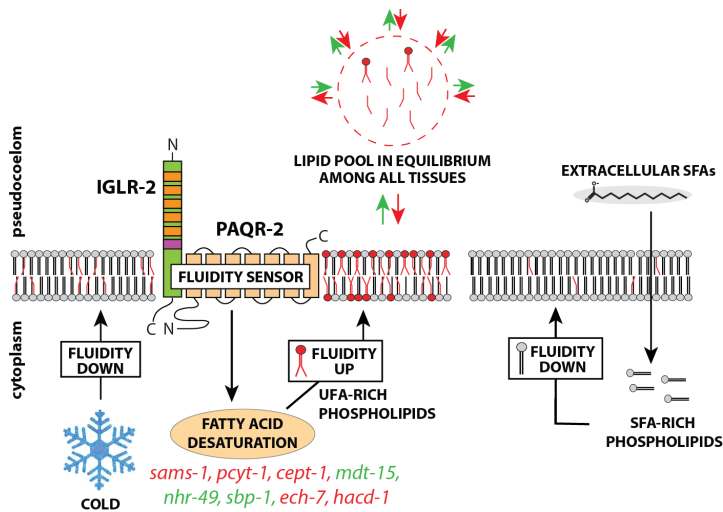
Fig. 11. PAQR-2/IGLR-2 regulates membrane homeostasis cell nonautonomously. PAQR-2/IGLR-2-mediated regulation of membrane homeostasis (as shown earlier in paper I and II) occurs cell non-autonomously (current study). PAQR-2/IGLR-2 expression in any large membranous tissues promotes fatty acid desaturation upon membrane rigidification, leading to systemic regulation of membrane homeostasis, a mechanism that relies on exchange on lipids among different tissues in *C. elegans*.

Own Contributions to Paper III

1. <u>Mosaic analysis experiments</u>: Co-injected the *SUR-5::GFP(NLS)* reporter with *iglr-2* and/or *paqr-2* rescuing transgene and performed mosaic analysis experiments.
2. <u>Constructs (Plasmids)</u>: Designed and generated gonad sheath cell specific construct for *paqr-2* (*Plim-7::PAQR-2::GFP)* and *fat-6 (Plim-7::FAT-6)*.
3. <u>Microinjection</u>: Injected and generated transgenic lines for gonad sheath cell and intestine specific transgenes.
4. <u>Growth experiments</u>: Tested the effect of different tissue specific transgenes on the growth in standard conditions, in the presence of glucose or at 15 °C.
5. <u>Genetic interaction studies</u>: Performed genetic interaction studies with *paqr-2* and *fat-6*/*fat7* mutants and further tested whether certain tissue specific transgene can compensate and rescue the phenotypes.
6. <u>FRAP experiments</u>: Performed and analyzed FRAP experiments to test whether specific expression of PAQR-2 in hypodermis can influence membrane fluidity in the intestine.
7. <u>Microscopy</u>: Acquired images to demonstrate the expression of FAT-6 in the hypodermis and the intestine.

PAPER IV: Membrane Fluidity is Regulated by the *C. elegans* Transmembrane Protein FLD-1 and its Human Homologs TLCD1/2

RESULTS

Mutations in *fld-1* can rescue growth defects in the presence of glucose

Previously, in Papers I and II, we showed that worms that lack a functional PAQR-2 or its partner IGLR-2 are sensitive to exogenous SFAs or glucose, the latter being converted to SFAs by the dietary *E. coli.* We also found that one efficient way to genetically counter this phenotype is through mutations in other genes and of which the effect is to promote fatty acid desaturation, a process that generates MUFAs and PUFAs and thus promotes membrane fluidity. Here, we again used forward genetics this time to isolate mutants that can rescue the lethality of *paqr-2* or *iglr-2* mutants on a glucose diet. Additionally, we also screened for mutations that further enhance the ability of *mdt-15(et14)* to suppress the glucose intolerance phenotype of *paqr-2* mutant, hence increasing the chance to isolate novel mutants that act independently from the desaturases, which are already activated by the *gof mdt-15(et14)* allele. We screened a total of over 100 000 haploid genomes and isolated 15 mutants that suppressed the growth defect of *paqr-2*, *iglr-2* and *paqr-2 mdt-15(et14)* mutants on a glucose diet. We outcrossed these novel mutants then had their genome sequenced, which revealed eight novel alleles of a previously uncharacterized gene that we dubbed *fld-1* (membrane **fl**u**id**ity homeostasis-1), as well as one novel allele of *paqr-1,* which will be described in detail in paper V, and one novel allele of *acs-13* (acyl-CoA synthetase-13) (215).

FLD-1 is localized to the plasma membrane

FLD-1 is a multi-pass transmembrane protein homologous to the mammalian TLCD1 and TLCD2, which belong to a family of proteins that includes ER localized ceramide synthases and translocation associated membrane proteins, and that are characterized by the presence of a TLC domain. The term TLC was coined after three members of this protein family: TRAM (translocation chain-associated membrane protein), LAG1P (an ER protein that facilitates transport of glycosylphosphatidylinositol-anchored protein from ER to Golgi) and CLN8 (an ER and Golgi resident protein) (216). TLCD1, also called "calfacilitin" localizes to the plasma membrane of mammalian cells and can facilitate the activity of at least one calcium channel (217). Using a translational GFP reporter (*Pfld-1::FLD-1::GFP*), we showed that FLD-1 is expressed on the plasma membrane of most, and perhaps all, cells in *C. elegans*. We also noticed that introducing a wild-type functional copy of *fld-1* in *paqr-2;fld-1* double mutant abolished the beneficial effect of *fld-1* mutation to grow on glucose, demonstrating that the *paqr-2* suppressor *fld-1* alleles are *lof* mutations.

FLD-1 acts independently of desaturases

We scored five of our eight novel alleles of *fld-1* for their ability to rescue three distinct high penetrance *paqr-2* mutant phenotypes: glucose intolerance, cold intolerance and the tail-tip morphology defect. We noticed that all tested *fld-1* alleles were similar in their ability to rescue the phenotypes, further suggesting that they are all likely *lof* alleles. We selected *fld-1(et48)*, which carries a mutation in a splice donor site, as the reference allele and used it throughout the rest of the mutant characterization experiments. It was interesting that we did not notice any visible phenotypes in the *fld-1* single mutant. In all of our experiments, including pharyngeal pumping, brood size, defecation, locomotion, survival at 30°C or 37°C, life span and ER-UPR activation, the *fld-1* single mutants were indistinguishable from wild-type worms. Further, *in vivo* FRAP measurements showed that the *fld-1 lof* mutation was also able to rescue the membrane fluidity defects observed in *paqr-2* mutant but that the single *fld-1* mutant had itself a normal membrane fluidity.

The fact that we were able to isolate *fld-1* mutants also from the *paqr-2 mdt-15(et14)* enhancer screen suggests that *fld-1* acts in a pathway distinct from the *gof* allele *mdt-15(et14)*. To further explore this, we performed genetic interaction studies with previously isolated *paqr-2* suppressors. We tested combination of the *paqr-2;fld-1(et48)* double mutant with: *mdt-15(et14)*-representative of the fatty acid desaturation pathway, *cept-1(et10)*-representative of the PC synthesis pathway, and *hacd-1(et12)*-representative of β-oxidation pathway, respectively, and found that the *fld-1(et48)* mutation could greatly enhance the *paqr-2* suppressor ability of all three suppressor mutations in terms of growth on glucose but could only enhance the effect of *mdt-15(et14)* and *cept-1(et10)* in terms of growth on PA-loaded *E. coli* (which is likely a stronger challenge). These results suggest that *fld-1(et48)* acts in a pathway distinct from that of *mdt-15(et14)* and *cept-1(et10)*, which we further confirmed by analyzing the *pfat-7::GFP* reporter. We found that the inability of *paqr-2* mutants to induce the expression of *pfat-7::GFP* was suppressed by *mdt-15(et14)* or *cept-1(et10)*, but not by the *fld-1(et48)* mutation. This strongly suggests that *fld-1(et48)* does not act by upregulating the expression of desaturases. However, RNAi of *fat-6* and *fat-7* abolished the rescue ability of *fld-1(et48)*, which suggest that the desaturation activity is nevertheless important for the ability of *fld-1(et48)* to rescue *paqr-2* mutant phenotypes.

Mutations in *fld-1* rescue the membrane defects by influencing PUFA levels

We further analyzed the fatty acid composition in PEs in worms cultivated under normal condition and when challenged with a glucose diet or with PA-loaded *E. coli*. As expected, we again noticed the increased accumulation of SFAs and reduced levels of PUFAs in the *paqr-2* mutant, and this was partially corrected by *mdt-15(et14)* as well as *fld-1(et48)* mutations in all the conditions. Interestingly, only the combination of *mdt-15(et14)* and *fld-1(et14)* provided a complete suppression of the FA composition phenotype, restoring their levels to wild-type levels. These results suggest that the *fld-1(et48)* and *mdt-15(et14)* mutations act in separate but complementary pathways to promote PUFA accumulation in membrane phospholipids at the expense of SFAs, and thus promote membrane fluidity.

The ability of *fld-1(et48)* to further enhance the accumulation of PUFA levels in *paqr-2 mdt-15(et14)* led us to speculate that the normal function of FLD-1 may be to limit the production of PUFA-containing phospholipids, or to promote their turnover. We therefore performed some genetic interaction studies with the *fat-2* mutant, which is not able to convert 18:1n-9 to 18:2n-6, the sole pathway for PUFA synthesis in *C. elegans* (29). We found that the *fld-1(et48)* mutation could slightly rescue the growth defect of the *fat-2* mutant on both normal condition and in the presence of glucose. Interestingly, the *fld-1(et48)* mutation could also efficiently rescue the glucose intolerance phenotype of *paqr-2;fat-2* double mutants. This is again consistent with the rescue ability of the *fld-1(et48)* mutation acting by promoting the accumulation of PUFA-containing phospholipids either by facilitating their production or by reducing their turnover rate, even though the actual *de novo* synthesis of PUFAs must actually be reduced in *paqr-2* or *fat-2* or *paqr-2;fat-2* mutants. This was further supported by the observation that the *fld-1(et48)* mutation suppressed the inability of *paqr-2* mutants to accumulate exogenously provided EPA. This last result, and the ability of *fld-1(et48)* to suppress *paqr-2* mutant phenotypes even when *fat-2* is mutated, strongly suggests that increased *de novo* synthesis of PUFAs is not the mechanism by which *fld-1(et48)* promotes the accumulation of PUFAs in membrane phospholipids. Rather, we propose that *fld-1(et48)* acts either by promoting the incorporation of any available PUFAs into phospholipids, or by reducing the turnover rate of phospholipids that contain PUFAs.

Mammalian TLCD1/2 regulate membrane composition and fluidity

Next, we examined whether the human homologs of FLD-1, i.e. TLCD1 and TLCD2, could also regulate membrane homeostasis in mammalian cells. We found that in HEK293 cells, the membrane rigidifying effects of PA could be suppressed by siRNA silencing of TLCD1/2, which by itself had little effect under normal conditions. We further explored different mechanisms that could possibly explain the protective effects of TLCD1/2 knockdown. Specifically, we found that the silencing of TLCD1/2 had no effect on the uptake of SFAs as measured using a labelled PA, which suggests that TLCD1/2 have no role in the uptake of SFAs. We also did not notice any change in the SFA content in the TAGs of PA-treated cells, which shows that TLCD1/2 do act by promoting the sequestration of PA into TAGs. Also, and consistent with the results from *C. elegans*, TLCD1/2 knockdown did not increase the levels of SCD or FADS2 mRNA. We further analyzed the lipid composition of cells treated with PA and found that the inhibition of TLCD1 markedly increased the levels of 18:2, 20:5 and 22:6 PUFAs in the PEs while inhibition of TLCD2 increased the levels of 18:2, 18:3 and 20:5 in the PCs. We did not notice any other striking changes induced by the inhibition of TLCD1/2 in the lipid composition of PA-treated cells; specifically, no change in the cholesterol levels or PC/PE ratio, and only a slight decrease in the total ceramide levels. Further, we cultivated cells with exogenous EPA and found that upon TLCD2 knockdown, such cells could accumulate more EPA in both their PCs and PEs. However, there was no change in the levels of EPA in TAGs and we did not notice any increase in the total TAGs, suggesting that TLCD2 does not regulate the uptake or storage of PUFAs. We also noticed, as others previously showed (218), that EPA is a highly potent membrane fluidizing fatty acid: concentrations as

low as 1 µM could rescue the membrane rigidifying effect induced by 400 µM PA. Therefore, a slight change in the PUFA levels in the membrane is highly significant. Altogether, these results convincingly show that increased PUFA levels in membrane phospholipids can be achieved by inhibiting TLCD1/2, and that this is beneficial and protective against the membrane-rigidifying effects of exogenous SFAs.

TLCD1/2 regulates the incorporation of PUFA-containing phospholipids

In order to test our hypothesis that wild-type FLD-1/TLCDs act either by limiting the incorporation of PUFAs into phospholipids or by promoting the turnover of PUFA-containing phospholipids, we cultured HEK293 cells in the presence of ^{13}C-labeled LA (18:2n-6) and examined its incorporation and clearance. We found that the inhibition of TLCD2 led to increased incorporation of ^{13}C-labeled LA in PCs and PEs as early as 6 hours of incubation and that the increased accumulation remained evident even after 24 hours in the PEs of TLCD1 and TLCD2 knockdown cells. However, we did not notice any changes in the chase part of the experiment, meaning the clearance rate of ^{13}C-labeled LA. These results suggest that functional TLCD1/2 normally act by limiting the incorporation of PUFA containing phospholipids but not by promoting their turnover.

We also confirmed that the inhibition of TLCD2 could rescue the PA-induced membrane rigidification in cells lacking AdipoR2, which is consistent with our results from worms where *fld-1(et48)* could rescue the membrane fluidity defects of the *paqr-2* mutant cultivated in the presence of glucose or fed PA-loaded *E. coli.* Further, both TLCD1 and TLCD2 knockdown suppressed the excessive SFA accumulation and reduced MUFA and PUFA levels observed in PA-treated cells lacking AdipoR2. Additionally, upon cultivating cells in 200 µM PA, we noticed increased ceramide levels in cells lacking AdipoR2, and this was normalized by TLCD1/2 knockdown. It is important to note that increased ceramide levels observed in cells lacking AdipoR2 is here consistent with the proposed ceramidase activity of AdipoRs (146, 147). In Paper II, we had noticed lower ceramide levels upon inhibition of AdipoR2 but the analysis in that paper was a result of culturing cells with 400 µM PA, which causes membrane composition defect even in wild-type cells (131).

DISCUSSION

As already mentioned, it is well known that dietary fatty acids have a significant impact in determining the fatty acid composition of the cellular membranes. Considering a huge variation in the types of dietary fatty acids, one can expect that cells must possess regulatory mechanisms to sense and respond to changes in membrane fatty acid composition. In this study, we have shown that FLD-1 in *C. elegans,* and TLCD1/2 in mammalian cells, limit the generation of PUFA-containing phospholipids. As a result, upon challenging worms or cells with exogenous or dietary SFAs, which induces membrane rigidification leading to cellular toxicity, mutations in *fld-1* or inhibition of TLCD1/2 is highly protective since it results in the increased accumulation of PUFAs in phospholipids, accompanied by improvement in membrane fluidity (Fig.12).

The FLD-1 and TLCD1/2 proteins are part of a protein family that includes ceramide synthases (CERSs) and translocation associated membrane proteins (TRAMs) (216). However, both CERSs and TRAMs are ER resident proteins while we and others (217) have found that FLD-1 and TLCD1/2 are localized to the plasma membrane. Further, FLD-1 and TLCD1/2 lack critical motifs found in ceramide synthases or translocation associated proteins and therefore are not likely to share either of these specific functions. Speculatively, the roles of FLD-1 and TLCD1/2 in regulating PUFA incorporation suggests that they could act on phospholipases or lysophospholipid acyltransferases, components of the Lands cycle pathway through which phospholipids are actively remodeled via fatty acid exchange (219). In the future, it would be interesting to pursue several genetic interaction studies with membrane bound acyl transferases in *C. elegans* to examine their possible role in relation to FLD-1 activity.
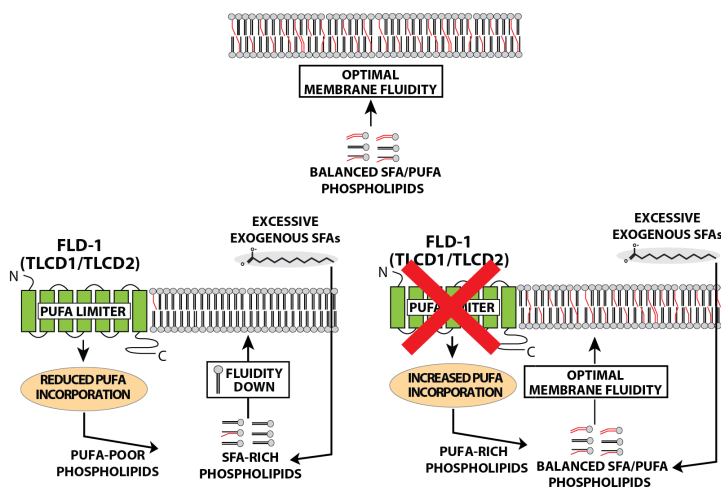

Fig. 12. FLD-1/TLCD1/2 regulate PUFA incorporation into membrane phospholipids. A healthy cell is able to achieve optimal membrane fluidity through balanced levels of SFA- and PUFA-containing phospholipids. When challenged with exogenous SFAs, the membrane rigidity temporarily increases due to increased proportion of SFA-rich phospholipids. Under these conditions, proteins such as FLD-1 or TLCD1/2 that normally function to limit the PUFA incorporation into membrane phospholipids, are detrimental to a cell since lower PUFA levels in combination with excessive SFA accumulation decrease membrane fluidity. Silencing of TLCD1/2, or mutations in FLD-1, especially under such membrane rigidifying situations, provides a beneficial and protective response since it leads to a new balance between SFA- and PUFA-containing phospholipids that restores membrane fluidity.

The incorporation of PUFAs into cell membranes is highly important to maintain membrane fluidity, cell permeability and hence influences complex physiological functions (6). However, the Achilles' heel of PUFAs, even though they are so important, is their susceptibility to lethal lipid peroxidation, a phenomenon also studied in *C. elegans* (220), that ultimately leads to the execution of ferroptosis, a form of regulated cell death characterized by iron-dependent

accumulation of lipid hydroperoxides to lethal levels (221). This explains the need for cells to have a regulatory machinery that limits the amount of PUFAs into membranes; the FLD-1 and TLCD1/2 may be parts of such a protective machinery.

To summarize, our results suggests a role for FLD-1 and TLCD1/2 in regulating PUFA levels in cellular membranes and we propose that inhibition of FLD-1 or TLCD1/2 is protective especially under membrane rigidifying conditions and therefore can be a potential therapeutic target in disease states linked to membrane rigidity such as diabetes (202) and perhaps also in cases of mutations in AdipoR1 that leads to retina defect because of the inability to accumulate DHA (199, 201).

Own Contributions to Paper IV

1. <u>Suppressor screen in *paqr-2* mutant background:</u> Performed a suppressor screen and isolated the *fld-1(et51)* allele.
2. <u>FRAP experiments</u>: Performed and analyzed all the FRAP experiments to test whether mutation in *fld-1* can rescue the membrane fluidity defects of the *paqr-2* mutant in various conditions.
3. <u>Lipidomics sample preparation</u>: Prepared all the lipidomics sample to survey the fatty acid profile in PEs in different genetic background and in different treatments.
4. <u>Genetic interaction studies with the *fat-2* mutant:</u> Performed genetic interaction studies with *paqr-2;fld-1* and *fat-2* mutant strains. Scored the growth on normal condition and in the presence of 20 mM glucose.

PAPER V: Leveraging A Gain-Of-Function Allele of *C. elegans* PAQR-1 to Elucidate Membrane Homeostasis by PAQR Proteins

RESULTS

A gain of function allele *paqr-1(et52)* compensates for the loss of *paqr-2*

    paqr-1(et52) is one of 15 mutant alleles that were isolated in a screen (described in Paper IV) for suppressors of the *paqr-2* mutant's glucose intolerance phenotype. The two PAQR proteins in *C. elegans*, namely PAQR-1 and PAQR-2, are quite similar with approximately 62% sequence identity in their transmembrane domain and almost 90% sequence identity in their localization motifs, previously reported to be important for receptor transport and localization (150, 222). However, they show much less sequence homology (25% identical) in the cytoplasmic N-terminal domain. The *paqr-1(et52)* mutation results in a R109C amino acid substitution in the N-terminal domain, which suggests that the non-conserved N-terminal domain of PAQR-1 likely possesses a regulatory function. We performed western blots against HA-tagged wild-type PAQR-1 and PAQR-1(R109C), generated using CRISPR/Cas9 modification of the endogenous locus, and found that the expression levels of PAQR-1 was not affected by the R109C mutation. Both PAQR-1 and PAQR-1(R109C) showed similar expression levels throughout development or when cultivated across different temperatures. Using a translational GFP reporter, we found that both PAQR-1 and PAQR-1(R109C) had similar sites of expression with a larger frequency of worms showing expression in the intestine and the gonad sheath cells, two tissues where PAQR-2 is also expressed and functional (164, 223). Further, introducing the *paqr-1(et52)* transgene in a *paqr-2* mutant background, which therefore still has an endogenous wild-type *paqr-1,* completely rescued the glucose intolerance, cold intolerance and tail-tip defect of the *paqr-2* mutant. This suggests that *paqr-1(et52)* likely acts as a *gof* allele that can functionally replace *paqr-2*. Additionally, we also noticed that overexpression of the wild-type *paqr-1* transgene (from a multi-copy extrachromosomal array) was also beneficial to the *paqr-2* mutant: it could rescue the cold intolerance and partially rescue the tail-tip phenotype of *paqr-2* mutant, though it did not rescue the glucose intolerance phenotype, suggesting a dose-dependent activity of wild-type PAQR-1 protein. Additionally, we found that substituting R109C with R109A also resulted in an equally effective *paqr-1 gof* allele, which rules out any role for cysteine-mediated disulfide bridges in the R109C allele and instead supports the hypothesis that the arginine residue at the 109 position plays a regulatory role in the wild-type protein. Altogether, these results suggest that while wild-type *paqr-1* possesses some basal activity that is more effective in higher doses, while *paqr-1(et52)* is a *gof* allele that is quite potent even when expressed at the same levels as wild-type endogenous *paqr-1*.

    We further characterized the *paqr-1(et52)* allele and found that the R109C mutation could partially or entirely rescue all the *paqr-2* mutant phenotypes including defects in pharyngeal pumping rate, brood size, lifespan, locomotion, defecation, glucose intolerance, cold intolerance and tail-tip defects. Further, the *paqr-1(et52)* mutation also corrected the excess SFA and reduced PUFA levels observed in the PEs of *paqr-2* mutants cultivated in the

presence of glucose or fed with PA-loaded *E. coli*. As a result, the increased membrane rigidity induced by a SFA-rich diet in the *paqr-2* mutant was also suppressed. These results suggest that *paqr-1(et52)* can act as a complete functional replacement for *paqr-2*. We further confirmed that the null *paqr-1(tm3262)* exacerbated several *paqr-2* mutant phenotypes such as poor growth, defects in membrane composition, brood size and locomotion rate, again supporting functional redundancy between these two proteins.

paqr-1(et52) is dependent on the same downstream effectors as *paqr-2*

As noted earlier, *gof* mutations in *nhr-49* or *mdt-15,* as well as overexpression of *sbp-1* can completely compensate for the loss of PAQR-2 during cold adaptation and that this is achieved at least in part via upregulation of Δ9 desaturase expression (38). This suggests that PAQR-2 likely regulates fatty acid desaturation via activating either *nhr-49* or *sbp-1,* with *mdt-15* being a co-factor for both of these transcription factors. If that is the case, then *paqr-1(et52)* might also act through *nhr-49, sbp-1* and/or *mdt-15* and the downstream desaturases, to act as a functional replacement in worms lacking *paqr-2*. Indeed, we found that RNAi against *mdt-15*, *sbp-1* and the *fat-5/-6/-7* desaturases abolished the *paqr-2* suppressor effects of the *paqr-1(et52)* mutation. Further, even though the synthetic lethality of the *paqr-2;nhr-49* double mutant (as reported in (37)) could be rescued by introducing a *paqr-1(et52)* mutation in this background, the resulting triple mutant could not be cultivated in the presence of glucose, suggesting that *paqr-1(et52)* does indeed depend on a functional *nhr-49* to mediate glucose tolerance.

paqr-1(et52) acts independently of *iglr-2*

We have shown previously that PAQR-2 and IGLR-2 are dependent on each other to maintain membrane homeostasis (164). To further explore whether *iglr-2* plays a role in mediating *paqr-1(et52)* activity, we performed some genetic interaction studies. We found that *paqr-1(et52)* was unable to rescue the glucose intolerance phenotype of the *iglr-2* mutant, even though it still suppressed its cold intolerance phenotype. Surprisingly, we also found that *paqr-1(et52)* was able to rescue both glucose and cold intolerance of the *paqr-2 iglr-2* double mutant. This interesting finding suggests that the presence of a functional *paqr-2* somehow interferes with the ability of *paqr-1(et52)* to suppress the phenotypes of the *iglr-2* single mutant and that *paqr-1(et52)* is not dependent on *iglr-2* to mediate its *paqr-2* suppressor effects. We further examined this by performing a BiFC experiment, where we did not notice any interaction between either wild-type PAQR-1 or PAQR-1(R109C) with IGLR-2. To summarize the genetic interaction studies, we found that *paqr-1(et52)* does not require *iglr-2* to rescue the *paqr-2* mutant phenotypes.

The intracellular N-terminal domain of PAQR-1 is inhibitory

That the *paqr-1(et52) gof* mutation results in a PAQR-1(R109C) amino acid substitution in the middle of cytoplasmic N-terminal domain suggests a potential regulatory function for

this domain that is relatively not well conserved between PAQR-1 and PAQR-2. To further understand the role of N-terminal domains in the function of the two proteins, we performed a series of experiments where we added an HA-tag immediately after the start codon at the N-terminus and swapped various protein domains between PAQR-1 and PAQR-2. Expression of all the resulting chimeric proteins was driven by the *paqr-2* promoter to allow direct comparisons. We tested the ability of each chimeric protein to rescue three distinct *paqr-2* mutant phenotypes: cold intolerance, glucose intolerance and the tail tip defect. The full length PAQR-1(R109C) completely rescued all the three phenotypes of both *paqr-2* and *paqr-2 iglr-2* double mutant. We did not notice any significant rescue with full length wild-type PAQR-1 expressed from the *paqr-2* promoter.

A chimeric protein consisting of the N-terminal domain of PAQR-1(R109C) fused with the transmembrane and C-terminal domains of PAQR-2 could only rescue the cold intolerance of *paqr-2* single mutant but not of the *paqr-2 iglr-2* double mutant. Further, this chimera also did not rescue the glucose intolerance or tail tip defect of the *paqr-2* single mutant. This suggest that a chimeric protein with N-terminal PAQR-1(R109C) and the transmembrane and C-terminal domains of PAQR-2 has less activity compared to wild-type full length PAQR-2 or full length PAQR-1(R109C) protein. Also, the results convey that it is the transmembrane domains of PAQR-2 that impose a requirement for the presence of IGLR-2 since this chimera could not rescue the phenotypes of *paqr-2* mutant worms that also lacked a functional *iglr-2* gene.

We also found that swapping of the extracellular C-terminal domain of PAQR-2 with that of PAQR-1(R109C) led to the reduction in the ability of the chimeric protein to rescue the phenotypes. This suggests that the activity of PAQR-1(R109C) is reduced when it harbors the C-terminal 44 amino acid residues of PAQR-2 rather than its own 5 amino acid residues.

Finally, a chimeric protein with the intracellular N-terminal domain of PAQR-2 fused to the transmembrane and C-terminal domains of PAQR-1 was as effective and efficient as the full length PAQR-1(R109C) mutant protein and could also act independently of IGLR-2. This is an interesting result suggesting that negating the effect of the inhibitory intracellular N-terminal domain of PAQR-1, either through the *paqr-1(et52)* mutation resulting in PAQR-1(R109C) or by replacing it with the N-terminal domain of PAQR-2, results in a constitutively active form of PAQR-1.

AdipoR1 overexpression compensates for the loss of AdipoR2

Next, we tested whether our observation regarding the overexpression of PAQR-1 being able to rescue the *paqr-2* mutant phenotypes is consistent with the mammalian cells. We found that overexpression of AdipoR1 or AdipoR2 in HEK293 cells could rescue the membrane rigidifying effects of 400 µM PA. This suggest that even in mammalian cells the AdipoRs act in a dose-dependent manner. We also noticed that the overexpression of AdipoR1 could rescue the membrane rigidification induced by 200 µM PA in cells lacking AdipoR2, consistent with the finding from *C. elegans* where the overexpression of PAQR-1 can compensate for the loss of PAQR-2 to some extent.

DISCUSSION

In this study, we isolated a *gof* allele of *paqr-1* and found that the resulting PAQR-1(R109C) protein is constitutively active and functionally able to compensate for the loss of PAQR-2. Our results also suggest that PAQR-1(R109C) is not dependent on IGLR-2 for its activity but nevertheless acts through the same downstream effectors as PAQR-2. Further, the structure-function studies have shown that it is the transmembrane domain of PAQR-2 that cause a dependency for the presence of IGLR-2 and, importantly, highlighted the regulatory function of the N-terminal domain of PAQR-1. Specifically, replacing the N-terminal domain of wild-type PAQR-1 with that of PAQR-2 creates a constitutively active chimeric protein. Finally, we could also show that overexpression of AdipoR1 in mammalian cells is beneficial just as with overexpression of the *C. elegans* PAQR-1, suggesting a dose-dependent activity of these proteins.

Our observation regarding the regulatory N-terminal region of PAQR proteins is consistent with the published report on the crystal structures of the AdipoRs, where the authors speculated that the small portion of N-terminal residues included in the study (residues 89-120 for AdipoR1) likely interfere and blocks the access of the substrates to the enzymatic cavity and that displacing it could increase access to the enzymatic cavity (145). This is somewhat analogous to the described 'ball and chain' or 'hinged lid' model proposed to regulate sodium channels in neurons where the cytoplasmic portion acts as a ball that can block, and therefore regulate, access to the channel (224, 225). In the future, it would be interesting to test whether the truncated proteins lacking the N-terminal domain can also be constitutively active.

Our findings regarding the overexpression of AdipoR1 or AdipoR2 being able to rescue the membrane rigidifying effects of PA are also consistent with published work showing that the inducible overexpression of the AdipoRs is sufficient to enhance the proposed ceramidase activity that further leads to improved glucose and lipid homeostasis (200). This clearly shows that, similarly to PAQR-1 in *C. elegans*, the mammalian AdipoRs can act in a dose-dependent manner.

While PAQR-1 acts independent of IGLR-2, PAQR-2 is completely dependent on, and interacts with, IGLR-2. We have previously speculated that conditions leading to increased membrane rigidity may promote the interaction between these two proteins, and in this way lead to their activation to further promote fatty acid desaturation and maintain membrane homeostasis (131, 164). In the present study, we noticed that it is the transmembrane domains of PAQR-2 that dictate a requirement for IGLR-2. This is not surprising since it is the transmembrane domains that are in the immediate vicinity of the lipid environment and can hence sense any aberration in the membrane lipid composition or properties. Speculatively, the regulatory consequence of the PAQR-2/IGLR-2 interaction likely occurs at the level of their cytoplasmic domains where it may lead to an activating conformational change, for example by displacing the cytoplasmic domain to increase access to the enzymatic cavity. In other words, the interaction of PAQR-2 with IGLR-2 through their transmembrane domains may result in the displacement of the cytoplasmic 'ball' that is postulated to regulate the access of substrates to the active site of PAQR-2 (Fig. 13).
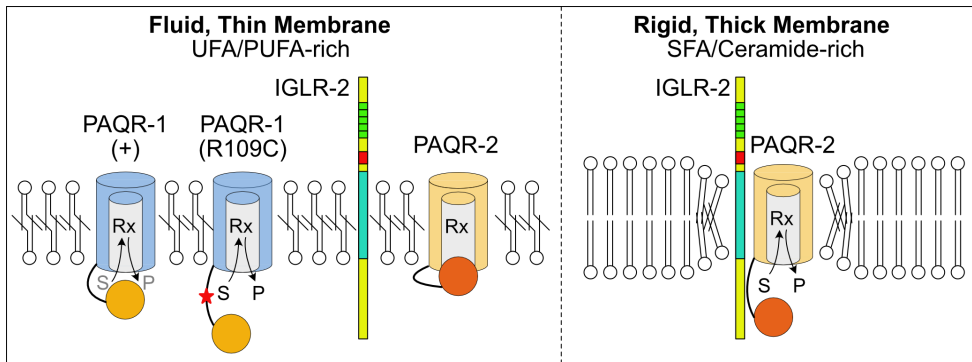
Fig. 13. Speculative model of PAQR-1/PAQR-2 regulation. A speculative model suggesting that the cytoplasmic N-terminal domain regulates the access of substrate (S) to the catalytic-site (Rx) in the transmembrane domain. In PAQR-1(+), the orientation of the intracellular domain always allows limited access of the substrate to be converted into product (P). In the PAQR-1(R109C) mutant protein, the intracellular domain is displaced, providing an unlimited access to the catalytic site that creates a constitutively active protein. In a fluid and thin membrane, both PAQR-2 and IGLR-2 are likely diffusedly distributed within the membrane bilayer with some occasional interaction. The cytoplasmic domain of PAQR-2 completely blocks the access of the substrate to the catalytic-site under these conditions. However, when the membranes are rigid and thick, PAQR-2 likely compresses the membrane around its vicinity, leading to formation of a stable complex with IGLR-2 that displaces the cytoplasmic domain and hence allows increased access to the catalytic site. The hydrolase (e.g. ceramidase) activity of PAQR-2 would then result in the production of a signaling molecule that will further activate the downstream effectors to promote fatty acid desaturation

Own Contributions to Paper V

1. <u>Microinjection:</u> Microinjected the BiFC constructs and translational reporters of PAQR-1 and PAQR-1(R109C) to generate transgenic lines.
2. <u>FRAP experiments:</u> Performed and analyzed FRAP experiment to test whether *paqr-1(et52)* can rescue the membrane fluidity defects of *paqr-2* mutant.

PAPER VI: AdipoR2 is Essential for Membrane Lipid Homeostasis in Response to Dietary Saturated Fats

RESULTS

paqr-2 and *iglr-2* are specifically essential to respond to dietary SFAs

In Paper I and Paper II, we showed that PAQR-2 and IGLR-2 are essential to tolerate dietary SFAs. However, it is an unsurprising possibility that there may exist many other types of proteins that could be required to counter the toxic effects of dietary SFAs. In order to more broadly understand and unravel the mechanisms by which *C. elegans* can tolerate dietary SFAs, we performed a forward genetic screen with a strategy to isolate mutants that cannot tolerate SFAs. We mutagenized wild-type N2 worms with EMS, singled-out the F1 progeny, presumed heterozygous for any novel mutation, individually picked and singled-out F2 progeny (a quarter of which are expected to be homozygous for any mutation present in the heterozygous state in F1s animal), further transferred the F3 progeny to a plate seeded with PA-loaded *E. coli* and, 72 hours later, screened for either lethality or sterility. For any well containing F3s unable to tolerate SFAs, the original F2 well was then tracked and the phenotype was re-confirmed (Fig. 14).
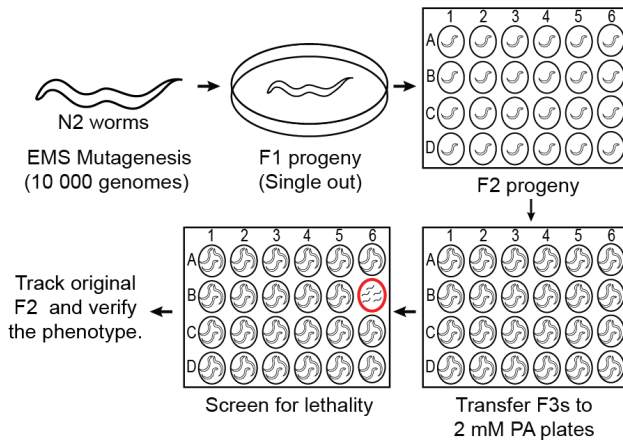
Fig. 14. Schematics showing how mutants that cannot tolerate dietary SFAs were isolated. Wild-type N2 worms were mutagenized with EMS, grown at 20°C and their F1 progeny were singled out into separate plates. The F2 progeny were then further individually picked and placed in 24-well plates. F2s were allowed to grow and 72 h later, the worms from the F3 generation from each well were transferred to plates seeded with PA-loaded *E. coli* OP50. These worms were allowed to grow and further screened for lethality or sterility (highlighted in red in the diagram). The source F2 well was then tracked and the phenotype was verified.

We rather laboriously screened a total of 10 000 haploid genomes and isolated five mutants that were completely intolerant of dietary SFAs. Upon further characterization, we found that when cultivated in PA-loaded *E. coli*, these mutants accumulated abnormally high levels of SFAs at the expense of MUFAs and PUFAs in their membrane phospholipids, which ultimately resulted in a marked reduction of membrane fluidity as measured using a FRAP assay. Further, after genetic complementation and genomic DNA sequencing of the candidate loci, we found that, astonishingly, four out of the five new mutants were novel *lof* alleles of *iglr-2* and the fifth mutant was a novel *lof* allele of *paqr-2*. This is an extremely significant result suggesting that in *C. elegans, paqr-2* and *iglr-2* are the only genes specifically essential for tolerance of dietary SFAs. It is important here to understand that genes that regulate some aspects of lipid metabolism and that are essential for survival even in normal conditions could not be isolated from this screening strategy, since those genes would cause lethality even in the absence of PA. However, this again provides an important conclusion that *paqr-2* and *iglr-2* are likely the only genes specifically required to respond to dietary SFAs and trigger an adaptive response. We further verified the identity of the mutations by introducing the wild-type copy of either *paqr-2* and *iglr-2*, as appropriate, which rescued the phenotypes. As expected the novel alleles of *paqr-2* and *iglr-2* were glucose intolerant, cold intolerant and also exhibited the characteristic tail-tip morphology defect. Again, and to emphasize our conclusion, these results show that *paqr-2* and *iglr-2* are the only genes specifically essential for tolerance of dietary SFAs in *C. elegans*.

Membrane properties influence the interaction between PAQR-2 and IGLR-2

We showed in Paper I and Paper V that PAQR-2 and IGLR-2 interact to form a complex in the intestinal membranes. We speculated then that their interaction may be directly regulated by membrane properties. Here, we investigated this hypothesis using the fluorescence resonance energy transfer (FRET) method (226). The FRET method is based on the principle that two fluorescent molecules that have significant overlapping spectra, if they are in close proximity (less than 10 nm), can transfer the emission energy from one molecule to excite the other, which can be recorded and quantified using a confocal microscope. The FRET method has been used as a powerful tool to visualize the interactions between proteins tagged with two fluorophores with overlapping excitation/emission spectra (227). Here, we tagged PAQR-2 with GFP and IGLR-2 with mCherry, a fluorophore combination previously used in FRET experiments (227–229), and found that the interaction between PAQR-2 and IGLR-2 on the membranes of the gonad sheath cells, a tissue where they are predominantly localized (164), was highly dynamic and was influenced by membrane properties. Specifically, we found increased FRET signal in a condition that promotes membrane rigidity (i.e. feeding with a SFA-rich diet) and conversely, we noticed that conditions that promote membrane fluidity (increased temperature or supplementing with mild-detergents or culturing on UFA-rich diet), decreased the interaction between PAQR-2 and IGLR-2. Unlike PAQR-2, we did not detect any FRET signal between PAQR-1 and IGLR-2, which is consistent with previous BiFC results discussed in Paper I and Paper V showing that these two proteins do not interact. These results suggest that the interaction between PAQR-2 and IGLR-2 is influenced by membrane composition and

it is therefore plausible that these proteins actually act as a sense-and-response protein system that is able to sense and respond to membrane saturation and rigidity by forming a complex that triggers an adaptive response that includes activation of fatty acid desaturases and restoration of membrane fluidity.

AdipoR2 knockout cells are extremely sensitive to SFAs

We showed in Papers II, III, IV and V that AdipoR2 in HEK293 cells and also in most other cell types (230), prevents membrane rigidification by modulating membrane composition and fluidity. Here, to explore and understand the overall consequences of AdipoR2 deficiency in cell physiology, we generated AdipoR2 knockout HEK293 cells using CRISPR/Cas9 by specifically deleting 23 amino acids in the N-terminus resulting in a frameshift mutation at R31. We then performed a series of experiments to characterize and verify the phenotypes of the knockout cells. We found that the AdipoR2-KO cells had delayed growth in basal condition and were extremely sensitive to PA supplementation. This effect could be rescued by the addition of small amounts of membrane-fluidizing EPA. We also measured the membrane fluidity using both the laurdan dye generalized polarization (GP) index and FRAP methods, and found, as expected, that the membranes of PA-treated AdipoR2-KO cells were rigid. The membrane rigidity observed in AdipoR2-KO cells could also be rescued by addition of EPA. Consistent with this, we observed that PA-treated AdipoR2-KO cells accumulated excess levels of SFAs at the expense of MUFAs and PUFAs in PCs and PEs and that this defect in membrane composition increased with the duration of PA treatment. We also found that AdipoR2 prevented membrane rigidification induced by other SFAs, such as MA or SA, suggesting that AdipoR2 prevents membrane rigidification induced by any SFAs, rather than acting specifically against the effects of PA. These results are consistent with our previous observation using siRNA inhibition and also show that the generated AdipoR2-KO is a true knockout cell line.

AdipoR2 is essential for a normal cellular response to PA

To explore and understand the overall consequences of AdipoR2 deficiency in cell physiology, we performed an RNA-seq experiment and analyzed the transcriptome of control and AdipoR2-KO cells grown in full media, serum-free media or serum-free media supplemented with PA for 0, 3, 9 and 24 hours. Overall, we monitored over 50 000 transcripts and observed a clear transcriptome difference among these treatments. We also noticed time-dependent increase in the abnormality of the transcriptomic response to PA treatment in AdipoR2-KO cells: 330 mis-regulated genes after 3 h, 1 310 after 9 h and 2 861 after 24 h of PA treatment. Of 3050 genes with q < 0.05 and at least 1.2-fold difference between control and AdipoR2-KO cells after 24 h of PA treatment, we found that 1556 genes were upregulated and 1494 were downregulated in AdipoR2-KO cells. Upon further analysis of the top 50 genes with the most significant variation among all treatments, we noticed that these genes clustered distinctly into four categories: 1) genes involved in fatty acid and cholesterol metabolism; 2) genes that similarly respond to starvation (either up or down) in both control and AdipoR2-KO cells; 3) genes that always differed (either up or down) between control and AdipoR2-KO cells;

and 4) genes involved in the ER-UPR. We also noticed that while the genes in the first category were severely downregulated, the genes in the fourth category were upregulated in PA-treated AdipoR2-KO cells. This suggest that maintenance of membrane homeostasis is the fundamental defect in AdipoR2-KO cells and is associated with failure to regulate unsaturated fatty acid and cholesterol synthesis in response to membrane rigidification. Further, it is most likely that the observed ER stress response is a secondary consequence of the primary membrane defect.

The SREBPs are well-known regulators of cholesterol and fatty acid metabolism (62). We found that, upon PA treatment, AdipoR2-KO cells transcriptionally phenocopied the loss of SREBPs. Experimentally, we also found that siRNA silencing of the SREBPs led to reduced expression of SCD, defect in membrane composition and increased membrane rigidity, these phenotypes being similar to those of PA-challenged AdipoR2-KO cells. This suggests the possibility that the observed downregulated genes in the SREBP pathway might contribute to the membrane defects in AdipoR2-KO cells. Further, we noticed that upon 24 h of PA treatment, the loss of AdipoR2 leads to the downregulation of pathways involved in respiratory electron transport, TCA cycle, unsaturated fatty acid biosynthesis, acyl chain remodeling, oxidative phosphorylation and glycolysis. Consistent with the transcriptomic results, the protein levels of SCD and FADS2 desaturases, the relative levels of cholesterol esters and mitochondrial respiration were all reduced in the AdipoR2-KO cells challenged with PA

AdipoR2 modulates membrane composition and fluidity to prevent SFA toxicity

Genes involved in fatty acid desaturation, such as SCD and FADS2, and genes involved in the incorporation of UFAs into cellular membranes, such as ACSL4 (acyl-CoA synthase ligase 4), are crucial for protecting cells against palmitate toxicity (231, 232). To directly compare AdipoR2 with these genes implicated in preventing palmitate toxicity, we used siRNA to silence AdipoR2, SCD, FADS2, ACSL4 and PEMT (phosphatidylethanolamine N-methyl transferase) in HEK293 cells and performed membrane fluidity assays and analyzed membrane lipid composition and the expression profile of the genes involved in the ER-UPR. Our results from a time-course FRAP experiment, showed that inhibition of AdipoR2, SCD and FADS2 led to membrane rigidification as early as 6 hours after PA treatment. Comparative lipid analysis measuring the levels of SFAs, MUFAs and PUFAs in PCs and PEs, PC/PE ratio, levels of lysophosphatidylcholine, free cholesterol, total TAGs and TAG 48:0 further strengthened our observation that silencing AdipoR2 or SCD had the most profound effect on lipid composition, followed by silencing of FADS2, ACSL4 and PEMT. Further, we noticed that membrane rigidification corelated with increased ER-UPR levels. These data confirm that AdipoR2 is a regulator of membrane homeostasis that is at least as important as other well-established anti-lipotoxicity genes involved in lipid metabolism.

Finally, we found that AdipoR2 also regulates membrane composition in primary cells: inhibition of AdipoR2 in primary human umbilical vein endothelial cells (HUVECs) led to the accumulation of membrane rigidifying SFAs and marked reduction of MUFAs and PUFAs when these cells are challenged with PA.

DISCUSSION

Here, we propose that diets rich in saturated fats lead to the synthesis of phospholipids that are rich in SFAs. Such SFA-rich phospholipids, when incorporated in the membrane bilayer, increase membrane rigidity. Unless cells have functional AdipoR2 (mammals) or PAQR-2/IGLR-2 (*C. elegans*), they will suffer from the toxic effects of membrane rigidification that will lead to ER stress and impaired mitochondrial function. When present, functional AdipoR2 or PAQR-2/IGLR-2 act as sense-and-response proteins that can activate the downstream fatty acid desaturases and other lipid metabolism genes to increases the levels of MUFAs and PUFAs in phospholipids, leading to an increase in membrane unsaturation and restoration of membrane fluidity levels compatible with a healthy cell (Fig.15).
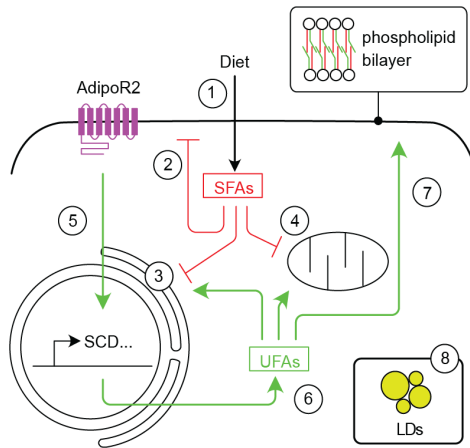

Fig. 15. AdipoR2 and membrane homeostasis in response to SFA-induced membrane rigidification. Diets rich in SFAs cause an excess of SFAs to be incorporated into membrane phospholipids (1) which could lead to membrane rigidification (2), ER stress (3), and impairment of mitochondria function (4). A healthy cell responds efficiently to any increase in membrane rigidification via AdipoR2-dependent activation of desaturases and other lipid metabolism genes (5), leading to increased levels of UFAs available for incorporation into phospholipids (6) and normalization of membrane fluidity (7). Some cells can also sequester SFAs into lipid droplets in the form of TAGs (8). A similar process occurs in *C. elegans*, where membrane rigidification activates the AdipoR homolog PAQR-2 by promoting its interaction with IGLR-2.

In this paper we probed for an answer to the question 'What is the mechanism to tolerate dietary SFA-toxicity?' by performing a forward genetic screen in *C. elegans* to isolate mutants sensitive to dietary SFAs. Our results suggest that PAQR-2 and IGLR-2 are the only sense-and-response proteins that are specifically essential for survival when worms are challenged with a SFA-rich diet. We showed that the interaction between PAQR-2 and IGLR-2 is directly modulated by membrane properties. By analogy to IRE1 in the ER membrane, which clusters

as the saturation and thickness of the membrane increases (73), we speculate that membrane saturation and rigidification that leads to an increase in membrane thickness also promotes the clustering, hence activation, of the PAQR-2 and IGLR-2 proteins. As mentioned earlier, clustering is thermodynamically favored in such conditions since it eliminates the requirement for each and every protein to locally deform the membrane in its vicinity in order to accommodate fixed-length transmembrane domains (73, 74). It seems likely that the PAQR-2/IGLR-2 complex acts as a plasma membrane fluidity sensor in eukaryotes analogous to IRE1 in the ER membrane (74), PCYT1 in the inner nuclear membrane (71) and DesK in the bacterial membrane (57).

The AdipoRs were initially proposed to act as receptors for adiponectin (132), a protein secreted by adipocytes. However, all of our experiments related to PA are performed in serum-free media and none of our experiments include adiponectin supplements. Further, HEK293 cells or the primary HUVECs that we studied do not express adiponectin (230), which is not surprising since it is an adipocyte-specific protein. Therefore, we can definitely say that AdipoR2-mediated regulation of membrane homeostasis can occur independently of adiponectin.

As mentioned earlier, SFAs-mediated lipotoxicity is an established contributor to the progression of many metabolic disorders (233). Excessive levels of SFAs cause apoptosis (234), increased ROS production (235) and also increased ER stress (236), which ultimately leads to cellular toxicity and cell death. In a recent genome-wide screen to understand palmitate-mediated cellular toxicity, it was reported that incorporation and accumulation of SFAs into membrane phospholipids is likely the toxic mediator of SFAs (231, 232). Consistently, loss of ACSL4, that promotes the incorporation of UFAs into membrane phospholipids increased the sensitivity to palmitate while loss of ACSL3, that promotes the incorporation of SFAs into membrane phospholipids, increased the resistance to palmitate (231). Interestingly, AdipoR2 ranked 4th in a genome-wide CRISPR/Cas9 screens to identify genes required to prevent inhibition of desaturase under hypoxic condition (237) and 25th in another screen aimed to identify genes important to prevent PA toxicity (231). This is consistent with our conclusion from *C. elegans* and mammalian cells that, the primary cellular function of the AdipoRs is to regulate membrane composition and fluidity.

Own Contributions to Paper VI

1. Performed the screen: Performed the screen to isolate mutants that are sensitive to dietary saturated fats. Different people have helped to complete this laborious screen.
2. Mutant characterization: Characterized the novel mutants isolated from the screen. Verified the mutation using wild-type transgenes, scored the growth in different conditions and scored the tail-tip phenotype.
3. FRAP experiments: Performed FRAP experiments to test the effect of dietary saturated fats on membrane fluidity in wild-type and mutants.
4. FRET experiments: Performed FRET experiments to test whether the interaction between PAQR-2 and IGLR-2 is modulated by membrane composition and fluidity.
5. Lipidomics sample preparation: Prepared all the worm lipidomics sample to survey the fatty acid profile in PCs and PEs in wild-type and mutants under different conditions.

CONCLUSION

To conclude, we have now documented that PAQR-2 in *C. elegans* and AdipoR2 in human cells are fundamentally important to regulate fatty acid desaturation/metabolism especially when worms or cells are challenged with saturated fats. In 2012, an entire study dedicated to understand the influence of dietary fats on membrane composition of rats reported, and I quote, "Diet SFA content had no influence whatsoever on membrane composition" (47). The authors concluded that there must exist a tight regulation to control the influence of dietary SFAs on membrane composition, though the mechanism was then unknown. We now believe that AdipoR2-mediated membrane homeostasis is the central mechanism that prevents excess SFAs from accumulating in phospholipids. The following points (below) and a graphical summary (Fig. 16) briefly highlight the most important findings from our studies so far, including two papers (2011 and 2013) published prior to my own PhD work:

2011: Characterization of *paqr-1*, *paqr-2* and *paqr-3*. *paqr-2* is the most important among the three genes. PAQR-2 is involved in fatty acid metabolism and cold adaptation (37).

2013: Forward genetics revealed that mutations that promote fatty acid desaturation can completely compensate for the loss of *paqr-2* during cold adaptation, thereby implying a role for PAQR-2 in fatty acid desaturation (38).

2016: Forward genetics identified *iglr-2* as a genocopier of *paqr-2*. The PAQR-2 protein interacts with IGLR-2 on plasma membranes, promotes fatty acid desaturation and hence modulates membrane lipid composition and fluidity to protect against glucose toxicity (**Paper I**).

2017: Glucose-toxicity in the *paqr-2* mutant is mediated by the bacteria. Bacteria converts glucose to SFAs via lipogenesis and it is the SFAs that are toxic to the *paqr-2* mutant. For the first time, we extended our findings in mammalian cells and found that PAQR-2 and AdipoR2 proteins share an evolutionary conserved function to maintain membrane homeostasis (**Paper II**).

2018: PAQR-2 and AdipoR2 regulate membrane homeostasis cell non-autonomously (**Paper III**).

2018: Forward genetics for suppressors of dietary SFA toxicity identified 8 alleles of *fld-1*. We found that FLD-1 in *C. elegans* and TLCD1/2 in mammalian cells limit the incorporation of PUFA-containing phospholipids in cellular membranes (**Paper IV**).

2020: The *gof* allele of *paqr-1(et52)* can completely compensate for the loss of *paqr-2*. Leveraging this *paqr-1(et52)* allele, we found that the intracellular N-terminal domain of PAQR-1 is inhibitory (**Paper V**).

2020: A forward genetics screen to identify genes required to tolerate saturated fats revealed that only *paqr-2* and *iglr-2* are specifically essential to respond to saturated fats. Further, the interaction between PAQR-2 and IGLR-2 is influenced by membrane properties and they likely act as a sensor of membrane fluidity. AdipoR2 in mammalian cells is important for a normal cellular response to saturated fats and protects against saturated fat-induced membrane rigidification by activating genes involved in fatty acid metabolism (**Paper VI**).
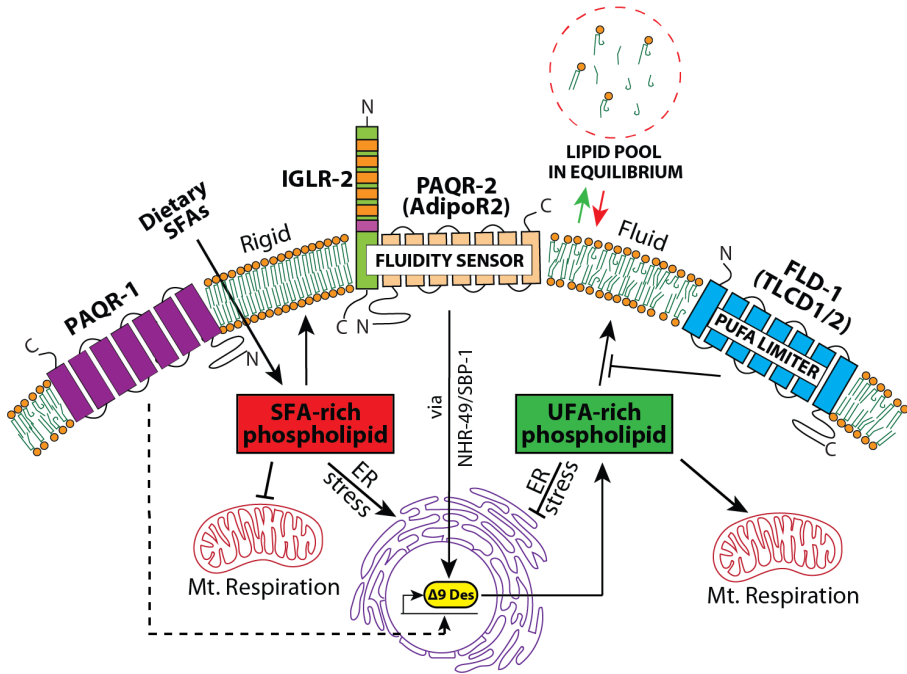

Fig. 16. Model summarizing membrane fluidity regulation: From *C. elegans* to mammalian cells. Dietary SFAs lead to the generation of SFA-rich phospholipids. In a healthy cell, the incorporation of these phospholipids in the lipid bilayer tend to increase membrane rigidity, which is immediately sensed by the PAQR-2/IGLR-2 fluidity sensor (in *C. elegans*) or AdipoR2 (in mammalian cells) that promote a compensatory downstream Δ9 desaturation (via activation of *nhr-49/sbp-1* in *C. elegans*). Δ9 desaturases convert SFAs to UFAs leading to the generation of UFA-rich phospholipids. The incorporation of these type of phospholipids in the membrane bilayer restore optimal membrane fluidity, which is important for normal cellular processes including mitochondrial respiration and proper ER function. Cells or mutants lacking PAQR-2/AdipoR2 function severely suffer from the membrane-rigidifying SFA-rich phospholipids, accompanied by increase in ER-UPR and impaired mitochondrial function. Other regulators such as FLD-1/TLCD1/2 limit the incorporation of UFA-rich phospholipids in the bilayer while PAQR-1 constantly promote fatty acid desaturation at a steady-state level. Further, an equilibrium of lipid pool is maintained between different cells/tissues, allowing cell non-autonomous regulation of membrane homeostasis.

MEMBRANE HOMEOSTASIS: SIGNIFICANCE AND IMPORTANCE

Initially cloned as the receptors for adiponectin and proposed as the mediators of adiponectin response, the AdipoRs have been extensively studied in mouse models to address their overall physiological function (132, 153–156). However, in the last decade, there has been a paradigm shift in our understanding of the primary cellular function of the AdipoRs: their primary role is in membrane homeostasis (11, 202, 230). Two recent forward genetics studies using CRISPR/Cas9 in human cells have further solidified the observation that AdipoRs are crucial for membrane homeostasis (231, 237). Specifically, AdipoR2 has ranked 4th out of nearly 20 000 genes tested for their ability to prevent inhibition of desaturase and membrane rigidification under hypoxic condition (237), and 25th out of all metabolic genes tested for their ability to prevent palmitate-induced cellular toxicity (231). These studies provide highly convincing support for the role of AdipoRs in maintaining membrane homeostasis. However, important "big picture" questions still remain. In particular: can this new view of AdipoR function help us better understand metabolic complications in disease states?

The integrity of biological membranes is fundamental for all cellular processes. Maintenance of overall homeostasis to achieve membrane heterogeneity within the cellular compartments is critical for processes such as vesicular trafficking, membrane fusion, exocytosis, endocytosis, etc. The activity and function of different receptors, transporters and channels implicated in many disease states are dependent upon specific lipid interactions (238). In particular, increase in membrane fluidity promotes insulin receptor signaling (239). Also, PUFAs, including EPA and AA, act as modulators of transient receptor channel V (TRPV) signaling (240). EPA and AA are also especially enriched in the membranes of photoreceptor cells for rapid flexibility to achieve desired receptor conformations (241). Lipid unsaturation in membrane bilayer also influence mitochondrial respiration (242), and membrane composition and fluidity similarly affects the dynamics and function of G-protein coupled receptors (GPCRs) (243, 244). These are only some of the examples where membrane composition/properties have been shown to directly influence vital signaling pathways within cells.

Therefore, it is not surprising that membrane composition and fluidity defects have been implicated in various metabolic disorders. Several disease states including diabetes (245–247), hypertension (248), Parkinson's disease (249, 250), Alzheimer's disease (251, 252), Huntington's disease (253, 254), retinitis pigmentosa (201, 255), and male sterility in mice (154, 256–258) have been correlated with defects in membrane properties. In fact, monitoring membrane fluidity in red blood cells has even been suggested as a diagnostic tool for evaluating the progression of diabetes (259). Altogether, these observations and results should suffice to convince any reader that maintenance of membrane fluidity and homeostasis is of paramount importance to the cell and, hence, that exploring pharmacological compounds to modulate the AdipoR pathway could be an important step going forward.

FUTURE PERSPECTIVES

We now know that the PAQR-2 and IGLR-2 proteins in *C. elegans* act as sense-and-response complex to prevent membrane rigidification by dietary SFAs. We also documented that upon membrane rigidifying conditions, mutations in *fld-1* or inhibition of TLCD1/2 allows incorporation and accumulation of membrane-fluidizing PUFAs in membrane phospholipids, acting as a compensatory mechanism to counter increased membrane saturation. These studies have partly been possible due to the strength of the unbiased forward genetics approach in *C. elegans*. However, the limitation of this approach is that the genes essential for survival under all conditions will be missed in most screening strategies. Therefore, in the future it would be interesting to use immunoprecipitation (IP) methods to identify the interacting partners of PAQR-2. Because of limitation in the availability of a robust PAQR-2 antibody, the endogenous *paqr-2* locus can be modified with a tag and IP-mass spectrometry experiments can be conducted using a tag-specific antibody. This approach would allow us to identify essential proteins that interact functionally with PAQR-2. Further, a similar approach can also be used with the mammalian AdipoRs. The IP-mass spectrometry approach would also allow to possibly identify and study eventual mammalian IGLR-2 homologs, if they exist.

Mutations in the genes that promote fatty acid desaturation can partly compensate for the loss of PAQR-2. In particular, we believe that *paqr-2* stimulates downstream desaturation via either *nhr-49* or *sbp-1*. However, we have not been able to narrow down the pathway to a single candidate so far. In the future, genetic interaction studies using tissue-specific transgenes of *nhr-49* or *sbp-1* along with *paqr-2* would be useful to identify unambiguously the actual effector. Different studies have also suggested that the AdipoRs act as ceramidases (141, 146, 147). If this is the case, it is an appealing hypothesis that one of the outputs from the ceramidase activity (i.e. a fatty acid molecule or S1P) could act as a ligand for the downstream *nhr-49* (or *sbp-1?*). Numerous fatty acids have been shown to act as a ligand for PPARs (161) and in particular, S1P has been shown to serve as an endogenous ligand for PPARγ (260). This could be an interesting avenue to explore since it could better define the PAQR-2/AdipoR2 signaling cascade to promote fatty acid desaturation.

Given the importance of membrane homeostasis in many disease contexts, it would be very interesting to carry out a study with a specific disease-relevant hypothesis in a specific cell-type. For example: Is AdipoR2-mediated membrane homeostasis important for insulin secretion in pancreatic β-cells? Given that glucose can be converted to SFAs via lipogenesis, it would be fascinating to know whether chronic high glucose treatment influences survival, proliferation and function of pancreatic β-cell. This would also provide information as to whether there exists a relationship between chronic hyperglycemia (a characteristic feature of diabetes) and membrane homeostasis.

In Paper III, we have shown that regulation of membrane homeostasis occurs cell non-autonomously. Specifically, we think that the lipids can be in equilibrium between different tissues and a healthy cell can systemically influence the robustness of an impaired neighboring cell. However, this hypothesis requires lipid carriers such as exosomes (261) that can transport lipid across tissues and therefore maintain overall membrane lipid homeostasis. It would be interesting to explore this in the future in both *C. elegans* and mammalian cells.

FLD-1 in *C. elegans* and TLCD1/2 in mammalian cells negatively regulates the incorporation of PUFA-containing phospholipids in the cellular membranes. How does this happen? What is the mechanism? We have speculated in our paper that *fld-1* may regulate phospholipid remodeling via the Lands cycle pathway to achieve this. The logical continuation of this work would be to explore this possibility either in *C. elegans* or mammalian cells. As mentioned earlier, the Achilles' heel of polyunsaturated fatty acids is their susceptibility to lethal lipid peroxidation that ultimately leads to the execution of ferroptosis, a form of regulated cell death characterized by iron-dependent accumulation of lipid hydroperoxides to lethal levels (221). This may explain why cells need a regulatory machinery to limit the amount of PUFAs into membranes for survival. The role of the TLCDs in preventing accumulation of lipid hydroperoxides could be further explored.

The *paqr-1(et52)* mutation resulting in the PAQR-1(R109C) protein can completely compensate for the loss of PAQR-2. We also found that the intracellular N-terminal domain of PAQR-1 is inhibitory and replacing it either with the N-terminal domain of PAQR-2 or modifying it with a PAQR-1(R109C) substitution mutation can also create a constitutively active protein. In the future, various N-terminal truncated constructs can be created and tested to see if they can also act as constitutively active proteins. The structural consequence of PAQR-1(R109C) can also be explored with molecular modeling approaches. Such computational methods could also be used to better understand the interaction between PAQR-2 and IGLR-2 in different membrane models.

Finally, a systematic compound/drug screen to identify modulators of the AdipoR pathway (using membrane fluidity as the output) could have wide implications in the medical field wherever membrane properties have medical relevance.

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